

Señor Director

DIRECCION DE NUEVAS CREACIONES

Superintendencia de Industria y Comercio

E. S. D.

Referencia: Expediente No. NC2016/0003727
Asunto: Solicitud de patente para ***“COMPOSICIONES PARA MODULAR LA EXPRESIÓN DE SOD-1”***
Solicitante: IONIS PHARMACEUTICALS, INC.
Fecha de solicitud: 01 de noviembre de 2016

RESPUESTA A EXAMEN PRELIMINAR

Yo, ANDRÉS RINCÓN USCÁTEGUI, identificado como aparece al pie de mi firma, en mi condición de apoderado de la sociedad solicitante de la patente de la referencia, doy respuesta al Oficio 13034, notificado por fijación en lista el 21 de noviembre de 2016, para lo cual pedí plazo adicional el 20 de diciembre de 2016.

I. MODIFICACIONES AL CAPÍTULO REIVINDICATORIO

Con el fin de atender las observaciones realizadas por la Examinadora de manera preliminar al examen de patentabilidad, adjunto un Capítulo Reivindicatorio Modificado conformado por 38 reivindicaciones en las que se han incluido las siguientes modificaciones:

1. Se eliminaron las antiguas Reivindicaciones 2 a 6, 23 a 30, 47 a 57, 59 a 67 y 69 a 83;
2. Se modificó la Reivindicación 1 de tal manera que se refiere particularmente a las SEQ ID NO: 725, 1173, 1342 y 1351, y tiene por característica el que tiene una secuencia de nucleobases que comprende al menos 12 nucleobases consecutivas de una de dichas secuencias. Esta modificación tiene soporte en la página 35, líneas 12 a 19, Modalidad 1, y en los Ejemplos 1, 2, 5, 9, 10, 11, 12,

“EXCELENCIA LEGAL EN UN MUNDO SIN FRONTERAS”

15 y Tablas 9, 15, 20, 21, 35, 40, 45, 50, 52, 54, 55, 56, 57, 59, 60, 66 del Capítulo Descriptivo;

3. Los porcentajes de complementariedad a los que ahora se refiere la nueva Reivindicación 2 (antigua Reivindicación 7) son *“al menos 90 % o 100%.”* Esta modificación tiene soporte en la página 38, líneas 5 a 13, Modalidad 6, y en los Ejemplos 1, 2, 5, 9, 10, 11, 12, 15 y Tablas 9, 15, 20, 21, 35, 40, 45, 50, 52, 54, 55, 56, 57, 59, 60, 66 del Capítulo Descriptivo.
4. Se eliminaron las expresiones *“8-10,” “4-6”* y *“5-7”* de la nueva Reivindicación 17 (antigua Reivindicación 22).
5. El preámbulo de las nuevas Reivindicaciones 18 a 33 ha sido modificado para referirse a un *“oligonucleótido modificado.”*
6. La nueva Reivindicación 35 corresponde a la antigua Reivindicación 58.
7. Se añadieron las nuevas Reivindicaciones 36 y 37, que se caracterizan por que el diluyente farmacéuticamente aceptable es solución salina amortiguada con fosfato (PBS) y por que la sal es una sal de sodio respectivamente, y tienen soporte la página 123, línea 4 a 18 y la página 123, línea 20 a la página 124, línea 8, respectivamente, del Capítulo Descriptivo.
8. Se añadió la nueva Reivindicación 38, que se caracteriza por que el nucleótido modificado es un compuesto antisentido conjugado, y tiene soporte en la página 124, línea 16 a la página 125, línea 2 del Capítulo Descriptivo;
9. Todas las reivindicaciones fueron reenumeradas, así como sus relaciones de dependencia, de acuerdo con todos los cambios anteriormente mencionados.

Estas modificaciones se ajustan al artículo 34 de la Decisión 486 de 2000, ya que no constituyen una ampliación de la protección que correspondería a la divulgación contenida en la solicitud inicial.

A pesar de eliminar materia del pliego reivindicatorio originalmente presentado, mi representada no renuncia a la protección que pueda obtener sobre dicha materia y se reserva el derecho de presentar una solicitud divisional que la contenga durante el trámite de la presente solicitud.

**II. EN CUANTO AL SOPORTE DE LAS REIVINDICACIONES, LA INCLUSIÓN DE
REIVINDICACIONES DE USO Y A LAS OBJECIONES RELACIONADAS CON EXCLUSIONES
A LA PATENTABILIDAD**

1. La Examinadora señala que:

- Las Reivindicaciones 1 y 2 no tienen sustento puesto que la descripción no da soporte sobre el hecho de que cualquier fragmento de 9 a 20 nucleobases de las SEQ ID NO 118 – 1461 tenga la actividad deseada. Así mismo, las Reivindicaciones 3 a 7 no tienen sustento ya que la descripción no muestra que cada una de las combinaciones de estructura mixta, motivos de unión a azúcar, o una secuencia que varíe en hasta un 20% en la secuencia tenga la actividad deseada.
- El objeto de las Reivindicaciones 64 y 65 no es patentable de acuerdo al Artículo 14, en tanto se refieren a un uso.
- El objeto de las Reivindicaciones 59 a 63 no es patentables de acuerdo con el Artículo 20 de la Decisión 486, toda vez que se refiere a métodos de tratamiento.

2. En respuesta a la objeción elevada por la Examinadora respecto al soporte de las reivindicaciones en el Capítulo Descriptivo, como se mencionó anteriormente, se modificó la Reivindicación 1, de tal manera que se refiere particularmente a las SEQ ID NO: 725, 1173, 1342 y 1351, y tiene por característica el que tiene una secuencia de nucleobases que comprende al menos 12 nucleobases consecutivas de una de dichas secuencias. Esta modificación tiene soporte en la página 35, líneas 12 a 19, Modalidad 1, y en los Ejemplos 1, 2, 5, 9, 10, 11, 12, 15 y Tablas 9, 15, 20, 21, 35, 40, 45, 50, 52, 54, 55, 56, 57, 59, 60, 66 del Capítulo Descriptivo. Adicionalmente, se eliminaron las antiguas Reivindicaciones 2 a 6.

Por último, como también se mencionó anteriormente, se modificó la nueva Reivindicación 2 (antigua Reivindicación 7) de tal forma que los porcentajes de complementariedad a los que ahora se refiere son “*al menos 90% o 100%,”* teniendo soporte en la página 38, líneas 5 a 13, Modalidad 6, y en los Ejemplos 1, 2, 5, 9, 10, 11,

12, 15 y Tablas 9, 15, 20, 21, 35, 40, 45, 50, 52, 54, 55, 56, 57, 59, 60, 66 del Capítulo Descriptivo.

De otro lado, difiero respetuosamente de la opinión de la Examinadora, en tanto considero que la invención no debería limitarse únicamente a los compuestos que son 100% complementarios al ácido nucleico objetivo. Respecto a ello, adjunto una copia de *“Activity of a Novel bcl-2/bcl-xL-Bispecific Antisense Oligonucleotide Against Tumors of Diverse Histologic Origins”*¹ (Anexo A), que revela el oligonucleótido 4625, el cual es 100% complementario a un primer ARNm objetivo (bcl-2) y 85% complementario a un segundo ARNm objetivo (bcl-xL). Dicho oligonucleótido ha demostrado ser capaz de inhibir la expresión de los dos ARNm en un 86% y 77%, respectivamente. Este artículo demuestra, por lo tanto, que oligonucleótidos con complementariedad menor al 90% respecto a un ARNm objetivo aún son capaces de inhibir su expresión. En este sentido, quisiera remitir a la Examinadora al Capítulo Descriptivo, página 73 línea 24 a página 74 línea 6, en donde se hace referencia a este artículo.

También anexo una copia de *“Specificity of antisense oligonucleotides in vivo”*² (Anexo B) que revela que es posible aumentar o disminuir el tamaño de un oligonucleótido, o aumentar el número de bases dentro del oligonucleótido que no son complementarias al ácido nucleico objetivo, sin perder actividad. En dicho artículo, se evaluó la habilidad de una serie de oligonucleótidos de 13 a 25 nucleobases en tamaño para inducir el corte del ARNm objetivo, en un modelo de inyección de ovocitos. Oligonucleótidos de 25 nucleobases en tamaño con 8 a 11 bases no complementarias cerca a los extremos de dichos oligonucleótidos fueron capaces de llevar a cabo el corte específico del ARNm objetivo, aunque en menor medida que aquellos oligonucleótidos que no contenían bases no complementarias. De manera similar, se logró un corte específico del objetivo usando oligonucleótidos de 13 nucleobases en tamaño, incluyendo aquellos con 1 a 3 bases no complementarias. Teniendo en cuenta las enseñanzas de Gautschi *et al.* y Woolf *et al.*, es evidente que, para la persona medianamente versada

¹ O. Gautschi et al., “Activity of a Novel Bcl-2/bcl-xL-Bispecific Antisense Oligonucleotide Against Tumors of Diverse Histologic Origins,” *JNCI Journal of the National Cancer Institute* 93, no. 6 (March 21, 2001): 463–71, doi:10.1093/jnci/93.6.463.

² T. M. Woolf, D. A. Melton, and C. G. Jennings, “Specificity of Antisense Oligonucleotides in Vivo.,” *Proceedings of the National Academy of Sciences* 89, no. 16 (August 15, 1992): 7305–9, doi:10.1073/pnas.89.16.7305.

en la materia, oligonucleótidos que difieren ligeramente en longitud o secuencia de aquellos contenidos en la solicitud serían todavía capaces de inhibir la expresión de SOD-1.

Adicionalmente, difiero respetuosamente de la opinión de la Examinadora respecto a que el Capítulo Descriptivo no muestra que cada una de las combinaciones de estructura mixta o motivos de unión a azúcar, pues dichas modalidades preferidas tienen soporte en, por ejemplo, las Tablas 45 y 46 del Ejemplo 11, las cuales se refieren a un número de compuestos de la secuencia reclamada en la Reivindicación 1 y distintos patrones de enlace internucleósido y modificaciones de azúcares.

Por todo lo anterior, considero superada la objeción en cuestión.

3. Respecto a las objeciones respecto a materia no patentable y reivindicaciones de uso, como se mencionó anteriormente, se eliminaron las Reivindicaciones 59 a 65 a las que hace referencia la Examinadora. En consecuencia, considero superadas las objeciones en cuestión.

4. Por todo lo anterior, considero que el Capítulo Reivindicatorio Modificado adjunto cumple con todos los requisitos de claridad y concisión, ajustándose así a los lineamientos del Artículo 30 de la Decisión 486, ya que cualquier persona medianamente versada en la materia está en completa capacidad de reproducir la invención sin ambigüedad alguna.

III. EN CUANTO AL PAGO DE TASA ADICIONAL POR EL NÚMERO DE REIVINDICACIONES

Presento junto con este escrito el comprobante de pago de tasa adicional para el estudio de 28 reivindicaciones adicionales.

IV. PETICIÓN

Teniendo en cuenta que la presente solicitud cumple con los requisitos de los Artículos 26 y 27 de la Decisión 486 de 2000 y considerando que se ha dado respuesta a cada una de las observaciones de la Dirección de Nuevas Creaciones con respecto a otros aspectos que tienen que ver con la patentabilidad de la invención, solicito a su Despacho continuar con el trámite administrativo correspondiente.

V. ANEXOS

1. Capítulo Reivindicatorio Modificado conformado por 38 reivindicaciones;
2. Comprobante de pago de tasa por reivindicaciones 28 adicionales;
3. Anexo A - O. Gautschi et al., "Activity of a Novel Bcl-2/bcl-xL-Bispecific Antisense Oligonucleotide Against Tumors of Diverse Histologic Origins," *JNCI Journal of the National Cancer Institute* 93, no. 6 (March 21, 2001): 463–71, doi:10.1093/jnci/93.6.463.;
4. Anexo B - T. M. Woolf, D. A. Melton, and C. G. Jennings, "Specificity of Antisense Oligonucleotides in Vivo.," *Proceedings of the National Academy of Sciences* 89, no. 16 (August 15, 1992): 7305–9, doi:10.1073/pnas.89.16.7305.

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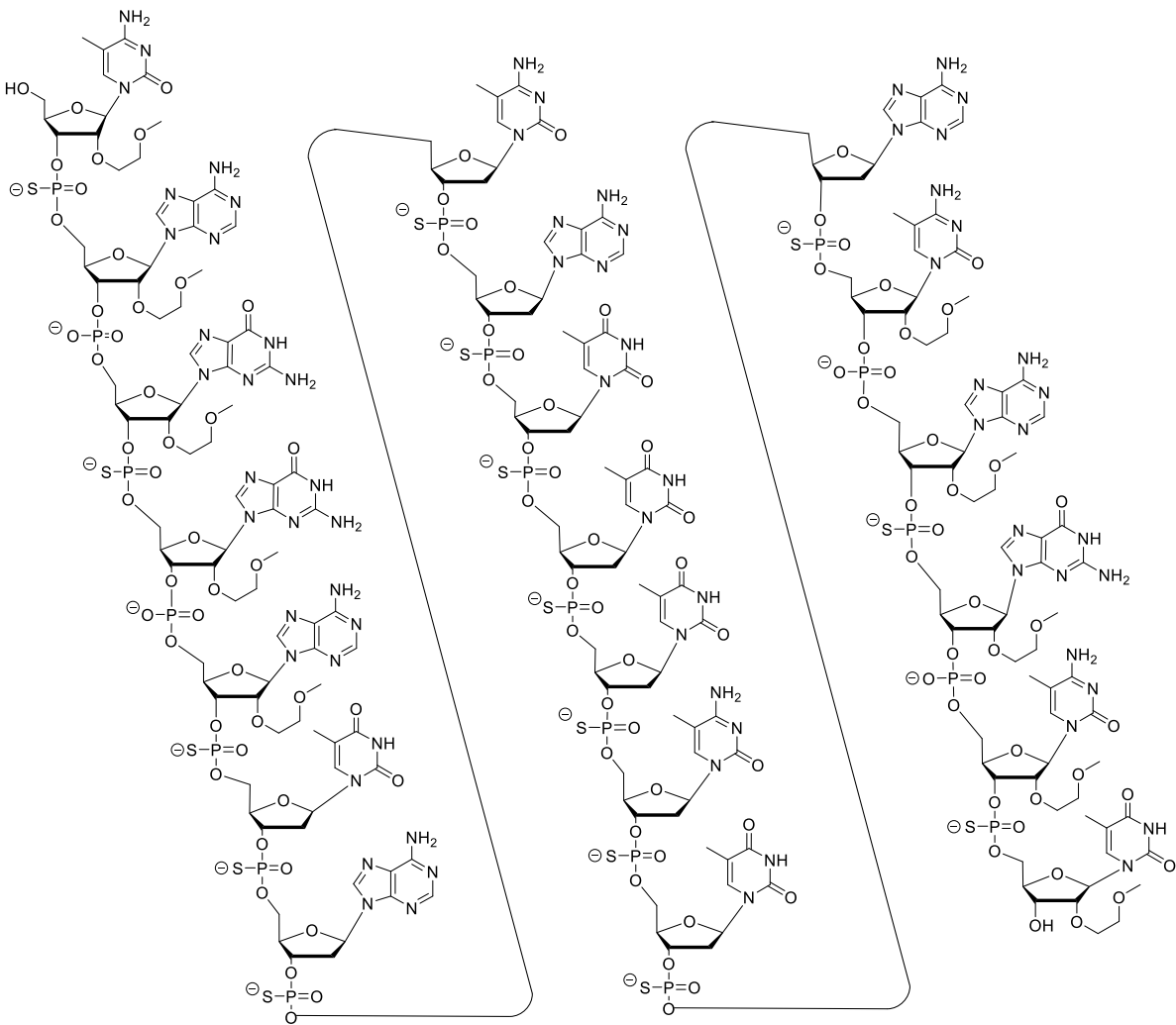
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CAPÍTULO REIVINDICATORIO MODIFICADO
(Examen de Forma)

1. Un compuesto que comprende un oligonucleótido modificado, que consiste en 12 a 30 nucleósidos enlazados y tiene una secuencia de nucleobases que comprende al menos 12 nucleobases consecutivas de cualquiera de las secuencias de nucleobases de las SEQ ID NO: 725, 1173, 1342 y 1351.
2. El compuesto de la Reivindicación 1, donde la secuencia de nucleobases del oligonucleótido modificado es al menos 90 % o 100 % complementaria con la SEQ ID NO: 1 y/o la SEQ ID NO: 2.
3. El compuesto de la Reivindicación 1 o 2, donde el oligonucleótido modificado es un oligonucleótido modificado de cadena simple.
4. El compuesto de cualquier reivindicación precedente, donde al menos una unión internucleósido es una unión internucleósido modificada.
5. El compuesto de la Reivindicación 4, donde al menos una unión internucleósido modificada es una unión internucleósido de fosforotioato.
6. El compuesto de la Reivindicación 5, donde cada unión internucleósido modificada es una unión internucleósido fosforotioato.
7. El compuesto de las Reivindicaciones 1 a 3, donde al menos una unión internucleósido es una unión internucleósido de fosfodiéster.
8. El compuesto de las Reivindicaciones 1 a 3, donde al menos una unión internucleósido es una unión de fosforotioato y al menos una unión internucleósido es una unión de fosfodiéster.
9. El compuesto de cualquier reivindicación precedente, donde al menos un nucleósido comprende una nucleobase modificada.

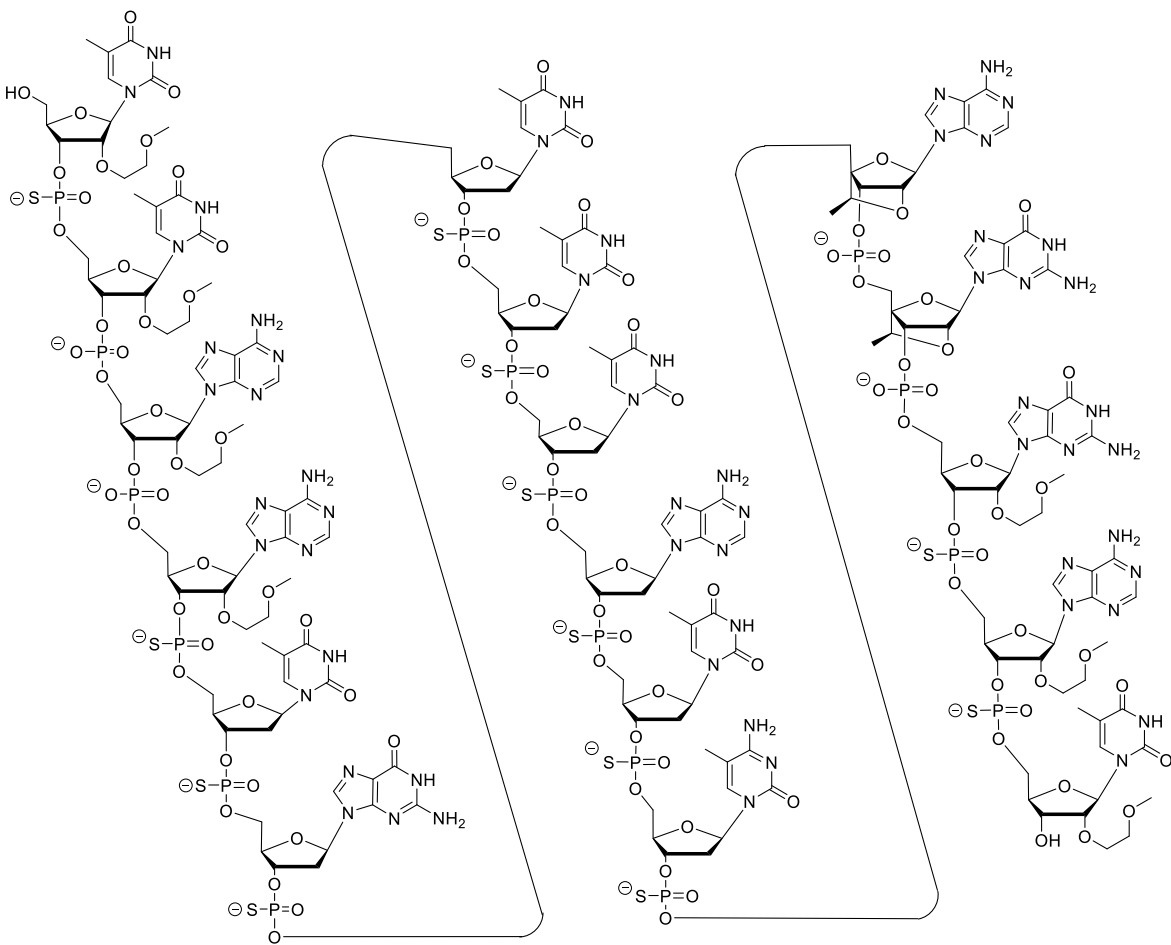
- 10.** El compuesto de la Reivindicación 9, donde la nucleobase modificada es una 5-metilcitosina.
- 11.** El compuesto de cualquier reivindicación precedente, donde al menos un nucleósido del oligonucleótido modificado comprende un azúcar modificado.
- 12.** El compuesto de la Reivindicación 11, donde el al menos un azúcar modificado es un azúcar bicíclico.
- 13.** El compuesto de la Reivindicación 12, donde el azúcar bicíclico comprende un puente 4'-CH(R)-O-2' donde R es, independientemente, H, alquilo C₁-C₁₂ o un grupo protector.
- 14.** El compuesto de la Reivindicación 13, donde R es metilo.
- 15.** El compuesto de la Reivindicación 13, donde R es H.
- 16.** El compuesto de la Reivindicación 11, donde el al menos un azúcar modificado comprende un grupo 2'-O-metoxietilo.
- 17.** El compuesto de cualquier reivindicación precedente, donde el oligonucleótido modificado comprende:
- un segmento de hueco que consiste en desoxinucleósidos enlazados;
 - un segmento de ala 5' que consiste en nucleósidos enlazados; y
 - un segmento de ala 3' que consiste en nucleósidos enlazados;
- donde el segmento de hueco está ubicado entre el segmento de ala 5' y el segmento de ala 3' y donde cada nucleósido de cada segmento de ala comprende un azúcar modificado.

18. Un oligonucleótido modificado de acuerdo con la siguiente fórmula:



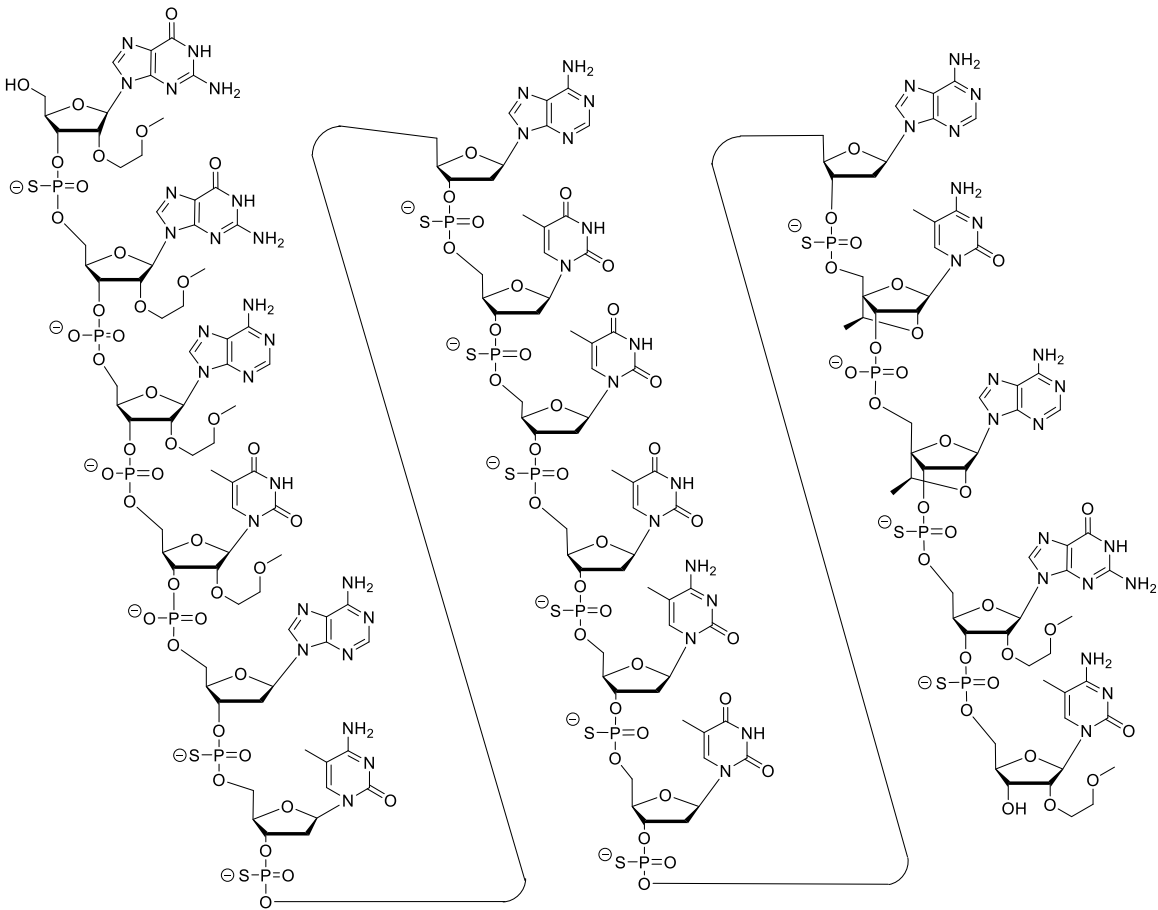
(SEQ ID NO: 725).

19. Un oligonucleótido modificado de acuerdo con la siguiente fórmula:



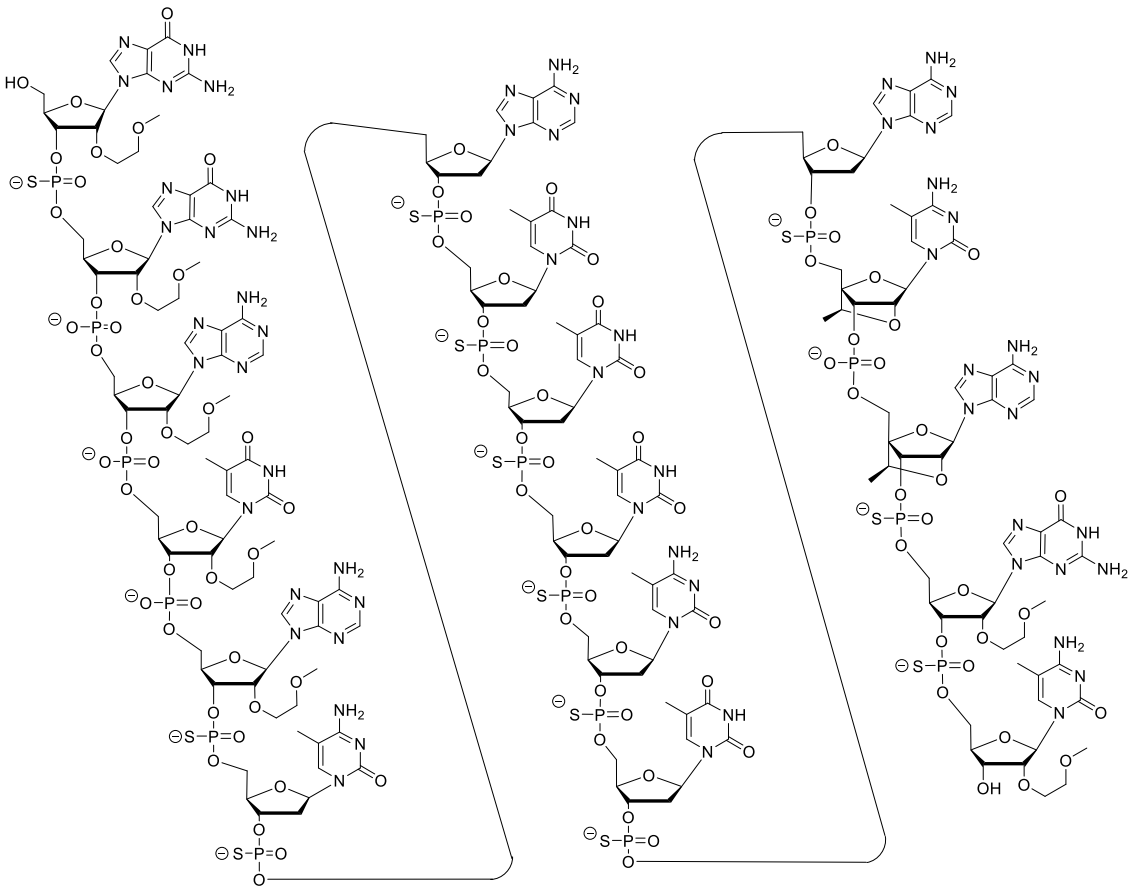
(SEQ ID NO: 1351).

20. Un oligonucleótido modificado de acuerdo con la siguiente fórmula:



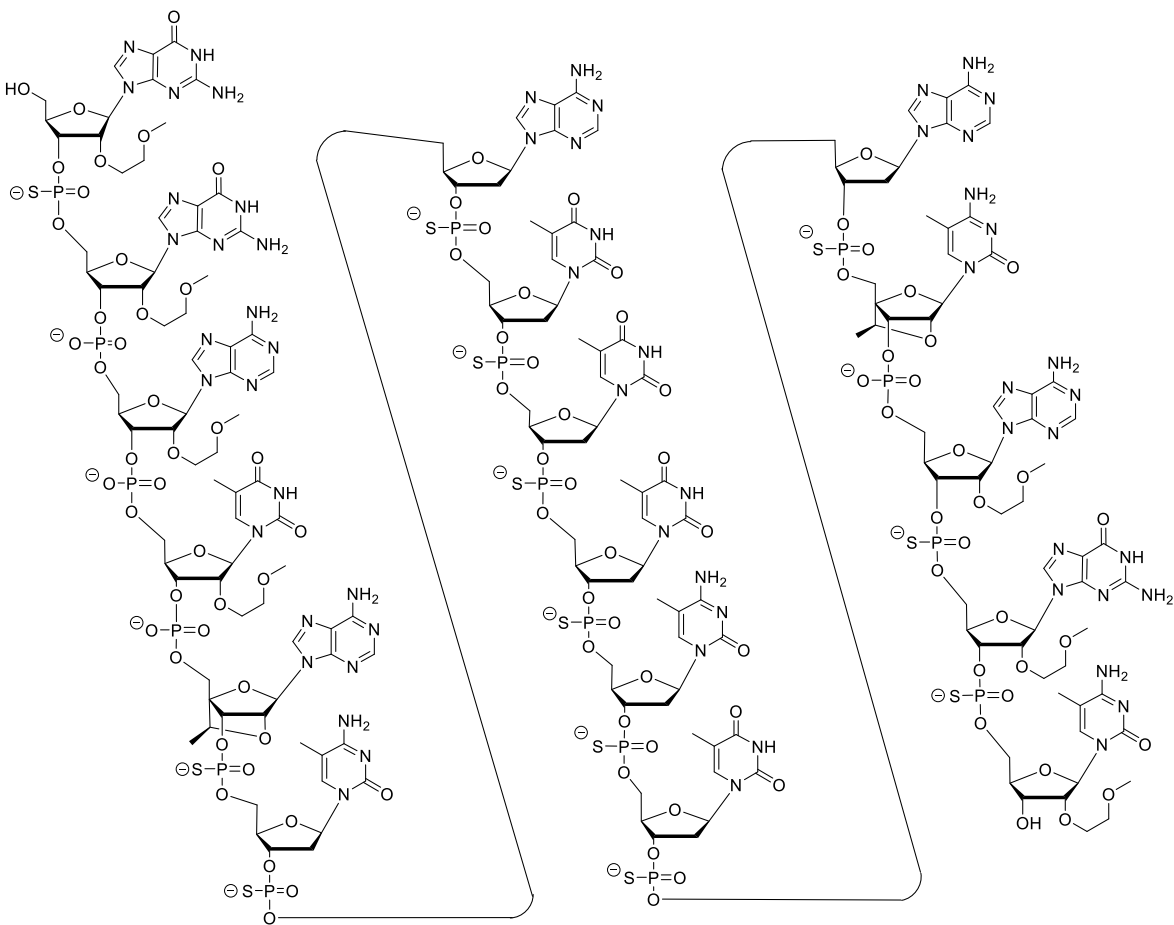
(SEQ ID NO: 1342).

21. Un oligonucleótido modificado de acuerdo con la siguiente fórmula:



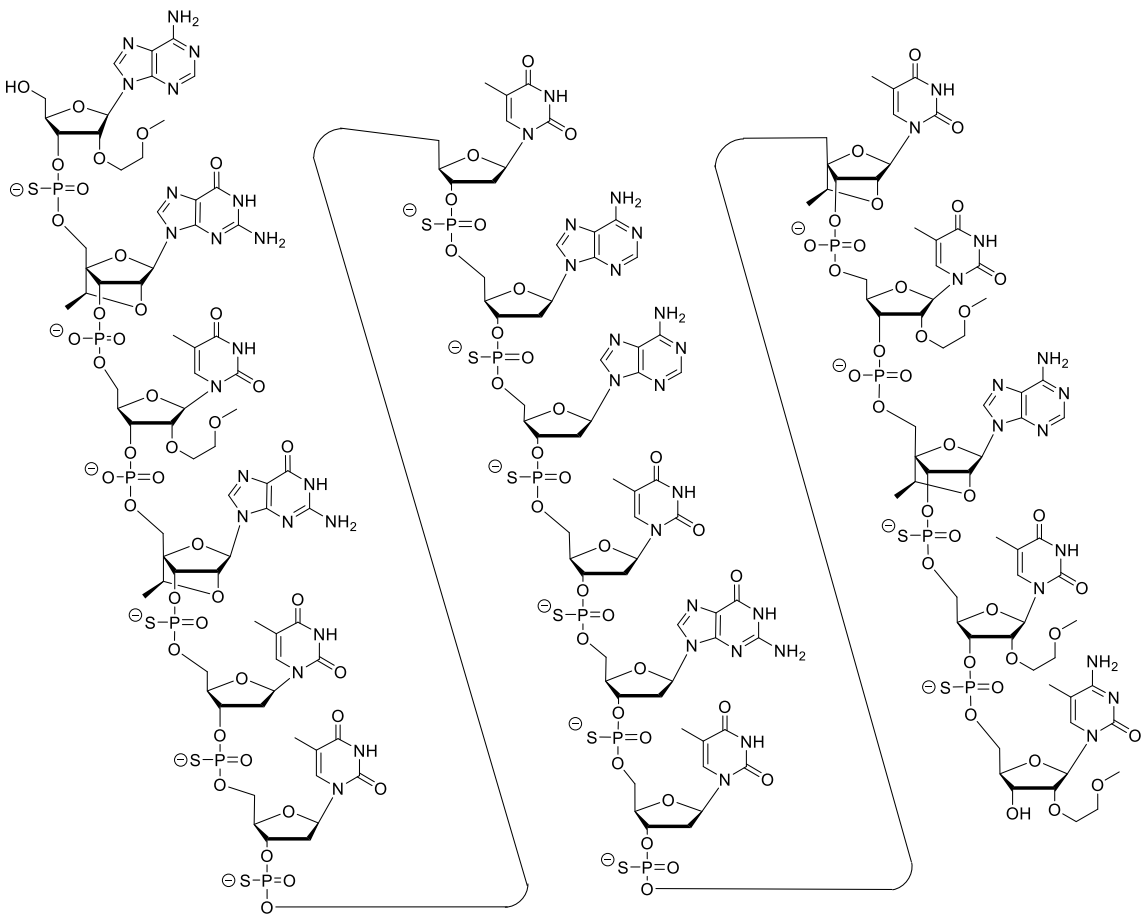
(SEQ ID NO: 1342).

22. Un oligonucleótido modificado de acuerdo con la siguiente fórmula:



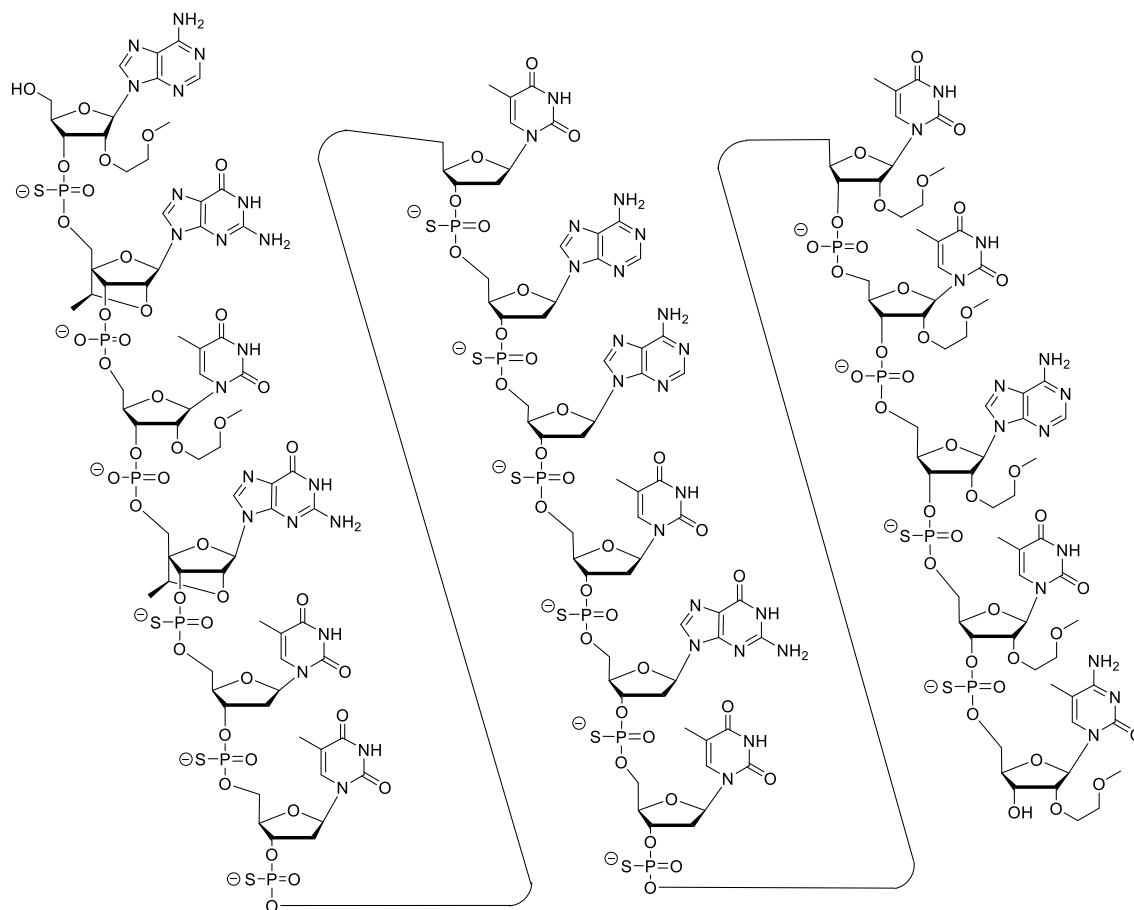
(SEQ ID NO: 1342).

23. Un oligonucleótido modificado de acuerdo con la siguiente fórmula:



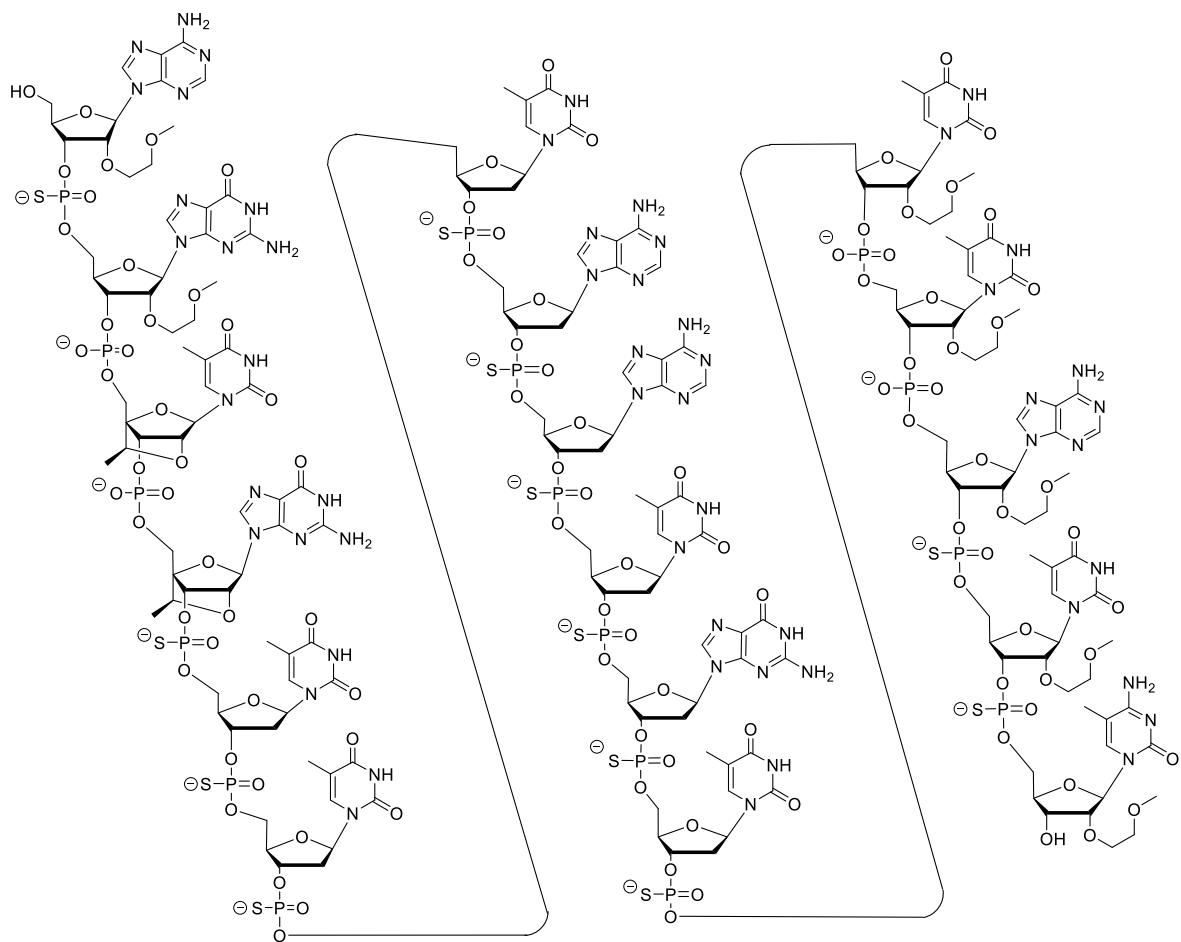
(SEQ ID NO: 1173).

24. Un oligonucleótido modificado de acuerdo con la siguiente fórmula:



(SEQ ID NO: 1173).

25. Un oligonucleótido modificado de acuerdo con la siguiente fórmula:



(SEQ ID NO: 1173).

26. Un oligonucleótido modificado de acuerdo con la siguiente fórmula:

mCes Aeo Ges Geo Aes Tds Ads mCds Ads Tds Tds Tds mCds Tds Ads mCeo Aes Geo mCes
Te; donde,

A = una adenina,

mC = una 5'-metilcitosina

G = una guanina,

T = una timina,

e = un azúcar modificado 2'-O-metoxietilribosa,

d = un azúcar 2'-desoxirribosa,

s = una unión internucleósido de fosforotioato, y

o = una unión internucleósido de fosfodiéster.

27. Un oligonucleótido modificado de acuerdo con la siguiente fórmula:

Tes Teo Aeo Aes Tds Gds Tds Tds Tds Ads Tds mCds Ako Gko Ges Aes Te; donde,

A = una adenina,

mC = una 5'-metilcitosina

G = una guanina,

T = una timina,

e = un azúcar modificado 2'-O-metoxietilribosa,

k = un azúcar cEt modificado,

d = un azúcar 2'-desoxirribosa,

s = una unión internucleósido de fosforotioato, y

o = una unión internucleósido de fosfodiéster.

28. Un oligonucleótido modificado de acuerdo con la siguiente fórmula:

Ges Geo Aeo Teo Ads mCds Ads Tds Tds Tds mCds Tds Ads mCko Aks Ges mCe; donde,

A = una adenina,

mC = una 5'-metilcitosina

G = una guanina,

T = una timina,

e = un azúcar modificado 2'-O-metoxietilribosa,

k = un azúcar cEt modificado,

d = un azúcar 2'-desoxirribosa,

s = una unión internucleósido de fosforotioato, y

o = una unión internucleósido de fosfodiéster.

29. Un oligonucleótido modificado de acuerdo con la siguiente fórmula:

Ges Geo Aeo Teo Aes mCds Ads Tds Tds Tds mCds Tds Ads mCko Aks Ges mCe; donde,

A = una adenina,

mC = una 5'-metilcitosina

G = una guanina,

T = una timina,

e = un azúcar modificado 2'-O-metoxietilribosa,

k = un azúcar cEt modificado,

d = un azúcar 2'-desoxirribosa,

s = una unión internucleósido de fosforotioato, y

o = una unión internucleósido de fosfodiéster.

30. Un oligonucleótido modificado de acuerdo con la siguiente fórmula:

Ges Geo Aeo Teo Aks mCds Ads Tds Tds Tds mCds Tds Ads mCko Aes Ges mCe; donde,

A = una adenina,

mC = una 5'-metilcitosina

G = una guanina,

T = una timina,

e = un azúcar modificado 2'-O-metoxietilribosa,

k = un azúcar cEt modificado,

d = un azúcar 2'-desoxirribosa,

s = una unión internucleósido de fosforotioato, y

o = una unión internucleósido de fosfodiéster.

31. Un oligonucleótido modificado de acuerdo con la siguiente fórmula:

Aes Gko Teo Gks Tds Tds Tds Ads Ads Tds Gds Tds Tko Teo Aks Tes mCe; donde,

A = una adenina,

mC = una 5'-metilcitosina

G = una guanina,

T = una timina,

e = un azúcar modificado 2'-O-metoxietilribosa,

k = un azúcar cEt modificado,

d = un azúcar 2'-desoxirribosa,

s = una unión internucleósido de fosforotioato, y

o = una unión internucleósido de fosfodiéster.

32. Un oligonucleótido modificado de acuerdo con la siguiente fórmula:

Aes Gko Teo Gks Tds Tds Tds Ads Ads Tds Gds Tds Teo Teo Aes Tes mCe; donde,

A = una adenina,

mC = una 5'-metilcitosina

G = una guanina,

T = una timina,

e = un azúcar modificado 2'-O-metoxietilribosa,

k = un azúcar cEt modificado,

d = un azúcar 2'-desoxirribosa,

s = una unión internucleósido de fosforotioato, y

o = una unión internucleósido de fosfodiéster.

- 33.** Un oligonucleótido modificado de acuerdo con la siguiente fórmula:
Aes Geo Tko Gks Tds Tds Tds Ads Ads Tds Gds Tds Teo Teo Aes Tes mCe; donde,
A = una adenina,
mC = una 5'-metilcitosina
G = una guanina,
T = una timina,
e = un azúcar modificado 2'-O-metoxietilribosa,
k = un azúcar cEt modificado,
d = un azúcar 2'-desoxirribosa,
s = una unión internucleósido de fosforotioato, y
o = una unión internucleósido de fosfodiéster.
- 34.** Un compuesto que comprende un oligonucleótido modificado que consiste en 12 a 30 nucleósidos enlazados y tiene una secuencia de nucleobases, donde la secuencia de nucleobases comprende un parte de al menos 12 nucleobases consecutivas complementarias a una cantidad igual de nucleobases de los nucleótidos 665 a 684 de la SEQ ID NO: 1, donde el oligonucleótido modificado es al menos 80 % complementario a la SEQ ID NO: 1.
- 35.** Una composición que comprende
el compuesto de cualquiera de las Reivindicaciones 1 a 17 o 34 o el oligonucleótido modificado de cualquiera de las Reivindicaciones 18 a 33 o la sal correspondiente, y
al menos uno de un portador o diluyente farmacéuticamente aceptable
- 36.** La composición según la Reivindicación 35, en donde el diluyente farmacéuticamente aceptable es solución salina amortiguada con fosfato (PBS).
- 37.** El oligonucleótido modificado de cualquiera de las Reivindicaciones 26 a 33, que es una sal de sodio de la fórmula.
- 38.** El compuesto de cualquiera de las Reivindicaciones 1 a 17 o 34 o el oligonucleótido modificado de cualquiera de las Reivindicaciones 26 a 33 o 37, donde el nucleótido modificado es un compuesto antisentido conjugado.

Activity of a Novel bcl-2/bcl-xL-Bispecific Antisense Oligonucleotide Against Tumors of Diverse Histologic Origins

Oliver Gautschi, Stefan Tschopp, Robert A. Olie, Siân H. Leech, A. Paula Simões-Wüst, Annemarie Ziegler, Bettina Baumann, Bernhard Odermatt, Jonathan Hall, Rolf A. Stahel, Uwe Zangemeister-Wittke

Background: Increased expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL is involved in the development and progression of many tumors. We recently reported that the bcl-2/bcl-xL-bispecific antisense oligonucleotide 4625 induces apoptosis in lung carcinoma cells. To further assess the therapeutic potential of oligonucleotide 4625, we investigated its effect on a series of human tumor cell lines of diverse histologic origins *in vitro* and *in vivo*. **Methods:** Oligonucleotide 4625-mediated inhibition of bcl-2 and bcl-xL expression *in vitro* was measured in breast carcinoma cells with the use of reverse transcription-polymerase chain reaction (PCR), real-time PCR, and western blotting. Cytotoxicity was assessed in several different cell lines by measurement of tumor cell growth, propidium iodide uptake, and nuclear apoptosis. The *in vivo* activity of oligonucleotide 4625 was determined by the inhibition of growth of established tumor xenografts in nude mice, immunohistochemical staining of Bcl-2 and Bcl-x proteins in the tumors, and western blotting of tumor lysates. Apoptosis in tumor xenografts was detected with the use of *in situ* TUNEL (i.e., terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick end labeling) staining. All statistical tests are two-sided. **Results:** In breast carcinoma cells, oligonucleotide 4625 treatment reduced bcl-2 and bcl-xL messenger RNA levels in a dose-dependent manner. At 600 nM, oligonucleotide 4625 reduced Bcl-2 and Bcl-xL protein levels to 25% (95% confidence interval [CI] = 16% to 34%) and 20% (95% CI = 14% to 26%), respectively, of the levels in untreated cells and it decreased viability in all cell lines mainly by inducing apoptosis. *In vivo*, oligonucleotide 4625 statistically significantly inhibited the growth of breast and colorectal carcinoma xenografts by 51% (95% CI = 28% to 74%) and 59% (95% CI = 44% to 74%), respectively, relative to those treated with control oligonucleotide 4626; it also reduced Bcl-2 and Bcl-xL protein levels and induced tumor cell apoptosis. **Conclusion:** The bcl-2/bcl-xL-bispecific antisense oligonucleotide 4625 merits further study as a novel compound for cancer therapy. [J Natl Cancer Inst 2001;93:463-71]

Impaired apoptosis due to blockade of the cell death-signaling pathways is involved in tumor initiation and progression, since apoptosis normally eliminates cells with damaged DNA or aberrant cell cycling, i.e., cells with increased malignant potential. From this recognition, a concept emerged that elevation of the threshold at which apoptosis is initiated represents a central step for tumor development and drug resistance. The structurally related Bcl-2 family proteins either inhibit or pro-

mote apoptosis, thereby regulating cell death in a rheostatic manner (1). The anti-apoptotic family members Bcl-2 and Bcl-xL both localize to cellular membranes, particularly in mitochondria, where they stabilize the transmembrane potential and reduce membrane permeability. This action inhibits the release from mitochondria of substances involved in either signaling or execution of apoptosis, such as cytochrome c, procaspase 3, and apoptosis-inducing factor (2). Although the issue is currently disputed, Bcl-xL may also inhibit apoptosis by binding to Apaf-1, thereby preventing its association with procaspase 9 and cytochrome c and the subsequent activation of downstream effector caspases (3). Although Bcl-2 and Bcl-xL seem to be functionally indistinguishable, there is evidence that they differ in their ability to protect cells from different apoptotic stimuli (4,5). In addition, Bcl-2 seems to contribute to tumor cell survival by diminishing the rate of cell proliferation (6) and by allowing tumor cells to escape from destruction by effector cells of the immune system (7).

The majority of solid tumors are protected by at least one of the two cell death antagonists, Bcl-2 or Bcl-xL. Although the relative expression levels of Bcl-2 and Bcl-xL vary, depending on the type and stage of the tumor, evidence for Bcl-2 and Bcl-xL overexpression as negative prognostic markers is provided by several findings: 1) Bcl-2 expression in non-small-cell lung carcinoma and estrogen receptor-positive breast carcinoma is associated with lymph node invasion (8,9); 2) Bcl-xL expression in breast and colorectal carcinomas is associated with advanced-stage disease (10,11); 3) Bcl-2 and Bcl-xL expression in prostate carcinoma is associated with tumor grade, metastatic potential, recurrence after prostatectomy, and radioresistance (12,13); and 4) overexpression of Bcl-2 in cutaneous malignant melanoma is associated with shorter survival (14). In tumors where Bcl-2 and Bcl-xL are coexpressed, it is difficult to predict which of the two proteins is more critical for survival and, thus, the more relevant target for gene therapy approaches. Moreover, tumor cells are able to switch expression from Bcl-2 to Bcl-xL (15), and forced overexpression of Bcl-2 has been reported to result in the reciprocal inhibition of Bcl-xL expression (16). In

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view of this complexity, the simultaneous inhibition of Bcl-2 and Bcl-xL expression in tumors by a single compound represents an appealing concept.

Antisense therapy holds the promise for selective silencing of genes, e.g., those involved in the resistance of cells to apoptosis induction, such as bcl-2 or bcl-x. Antisense oligonucleotides act by specifically hybridizing with complementary messenger RNA (mRNA) sequences based on Watson-Crick base pairing, thereby inhibiting gene expression primarily by activation of ribonuclease (RNase) H, which subsequently cleaves the target mRNA (17). Second-generation antisense oligonucleotides with a phosphorothioate backbone and up to five nucleotides at both the 5' and the 3' end modified by methoxy-ethoxy (MOE) residues at the 2'- α -position of the deoxyribose show enhanced RNA-binding affinity compared with their first-generation 2'-deoxy counterparts. Moreover, owing to the central 2'-deoxy gap, these antisense compounds are still capable of activating RNase H (18). Antisense oligonucleotides complementary to either the bcl-2 or the bcl-xL mRNA have previously demonstrated activity against tumor cells of various origins (19–24). Bcl-2 antisense oligonucleotides in combination with chemotherapy are currently in phase I/II clinical trials for the treatment of patients with lymphoma and malignant melanoma, and further trials with patients with lung, prostate, renal, or breast carcinoma are ongoing or planned.

Based on the assumption that simultaneous inhibition of bcl-2 and bcl-xL expression with a single compound might be therapeutically more promising than the inhibition of either alone, we have recently designed the bcl-2/bcl-xL-bispecific antisense oligonucleotide 4625 and reported its ability to inhibit the expression of bcl-2 and bcl-xL and to induce apoptosis in lung carcinoma cells *in vitro* (25). In the present study, we investigated the ability of antisense oligonucleotide 4625 to inhibit the expression of bcl-2 and bcl-xL in a representative breast carcinoma cell line and to inhibit the growth and induce death in cell lines from lung, breast, colorectal, and prostate carcinomas and from malignant melanoma *in vitro*. In addition, the ability of oligonucleotide 4625 to inhibit tumor growth and to reduce the Bcl-2 and Bcl-xL proteins *in vivo* was examined in established tumor xenografts in nude mice. Our data demonstrate the potent anti-tumor activity of oligonucleotide 4625 against a series of tumor cell lines *in vitro* and *in vivo* and suggest its use in cancer therapy.

MATERIALS AND METHODS

Cell Lines

The following nine human tumor cell lines of different histologic origins were obtained from commercial cell culture collections unless indicated otherwise: SW2 (small-cell lung carcinoma), NCI-H125 (non-small-cell lung carcinoma), MDA-MB-231 (estrogen-independent breast adenocarcinoma), MCF-7 (estrogen-dependent breast adenocarcinoma), Colo-320 (colorectal adenocarcinoma), LoVo (colorectal adenocarcinoma), PC-3 (prostate adenocarcinoma), C4-2 (prostate adenocarcinoma; provided by Dr. G. Thalmann, University Hospital, Bern, Switzerland), and A-375 (cutaneous malignant melanoma; provided by Dr. R. Dummer, University Hospital, Zurich, Switzerland). MDA-MB-231 cells were cultured in high-glucose Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 IU/mL penicillin, and 50 μ g/mL streptomycin. All other cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 2.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, 50 IU/mL penicillin, and 50 μ g/mL streptomycin. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. All cell culture reagents were obtained from Hyclone Europe, Ltd. (Cramlington, U.K.).

Oligonucleotides

Antisense oligonucleotide 4625 binds to a region of high homology shared by bcl-2 and bcl-xL comprising nucleotides 605–624 and 687–706 of the two mRNAs, respectively (25). These target sites are located 573 and 550 nucleotides downstream of the AUG start site of the bcl-2 and bcl-xL mRNA, respectively. Oligonucleotides 4626 and 4258 were used as scrambled sequence controls. All oligonucleotides were 20-mers with a phosphorothioate backbone and nucleotides at the 5' and 3' ends modified by MOE residues at the 2'- α -position of the deoxyribose (represented by underlined letters in the sequences shown below). Oligonucleotides were synthesized as described previously (26) on an Oligopilot II (Amersham Pharmacia Biotech, Uppsala, Sweden) at a 150- to 180- μ mol scale on a polystyrene primer (Amersham Pharmacia Biotech) derivatized via a succinyl arm by the first corresponding 2'-MOE 3' nucleoside. Crude oligonucleotides were purified by reverse-phase high-performance liquid chromatography. Purity was assessed by capillary gel electrophoresis, the phosphodiester content was checked with ³¹P-nuclear magnetic resonance, and the oligonucleotides were characterized by MALDI-TOF MS (i.e., matrix-assisted laser desorption ionization time of flight mass spectrometry). Oligonucleotides displayed a length purity higher than 95% with a phosphodiester content of less than 0.3%. The sequences were as follows: 4625, 5'-AAGGCATCCAGCCTCCGTT-3' (antisense); 4626, 5'-CACGTCACGCGCGCACTATT-3' (control); and 4258, 5'-CATATCACGCGCGCACTATG-3' (control).

Treatment of Cells With Oligonucleotides

In vitro, oligonucleotides were delivered to the cells in the form of complexes with the transfection reagent Lipofectin (Life Technologies, Inc. [GIBCO BRL], Glasgow, U.K.) as described previously (25). Lipofectin (50 μ g/mL) was allowed to complex with oligonucleotides, and this solution was further diluted to the desired concentration in serum and antibiotic-free medium before addition to the cells. Except for the growth assays described below, cells were incubated with oligonucleotides and Lipofectin for 20 hours, after which the Lipofectin solution was replaced by standard cell culture medium as described above.

Reverse Transcription-Polymerase Chain Reaction

One microgram of total RNA was reversed transcribed for 1 hour at 37°C in 20- μ L reactions containing 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 500 μ M each deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), and deoxythymidine triphosphate (dTTP), 7.5 μ g/mL random hexamers (Promega Corp., Madison, WI), 10 mM dithiothreitol, and 200 U Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). For the detection of bcl-2 mRNA, 2 μ L of the reverse-transcribed material was amplified by polymerase chain reaction (PCR) in 50- μ L reactions containing 10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 200 μ M each dATP, dGTP, dCTP, and dTTP, 0.2 μ M of each primer, and 0.25 U AmpliTaq DNA polymerase (Applied Biosystems [formerly Perkin-Elmer Applied Biosystems], Foster City, CA), for a total of 30 cycles consisting of 94°C for 45 seconds, 55°C for 60 seconds, and 72°C for 60 seconds, with a final extension step for 10 minutes at 72°C. Detection of bcl-xL mRNA was done under similar conditions, except that 1.5 mM MgCl₂ was used in the PCR reaction, and cycles were reduced to 28. To control for the presence and quantity of RNA in each reaction, we reamplified 5 μ L of each PCR for 33 cycles under similar conditions with primers for β 2-microglobulin able to discriminate between complementary DNA (cDNA) and genomic DNA. Amplification products were analyzed by electrophoresis in ethidium bromide-stained agarose gels. Primer sequences were as follows: bcl-2, 5'-GTGAAGTGGGGAGGATTGT-3' (forward) and 5'-GGAGAAATCAAACAGAGGCC-3' (reverse); bcl-xL, 5'-CCCAGAAAGGATACAGCTGG-3' (forward) and 5'-GCGATCCGACTCACCAATAC-3' (reverse); and β 2-microglobulin, 5'-GTGGAGCAT TCAGACTTGTCTTTCAGC-3' (forward) and 5'-TTCACCTAATCCAAATGCGGCATCTTC-3' (reverse).

Real-Time PCR

Total RNA was isolated from cells by use of the RNeasy Mini Kit (Qiagen AG, Basel, Switzerland). For cDNA synthesis, Taqman reverse transcription reagents were used as described in the user's manual of the ABI Prism 7700 Sequence Detection system, which was used for real-time PCR amplification following the Taqman Universal PCR Master Mix protocol (Perkin-Elmer Applied Biosystems). Relative quantification of gene expression was performed as

described in the manual with the use of ribosomal RNA (rRNA) as an internal standard and the comparative threshold cycle method. Briefly, 0.5 μ g of total RNA was used to generate cDNA by the reverse transcription reaction. The bcl-2 and bcl-xL cDNAs were amplified with the use of primers and Taqman probes that had been selected with the use of the Primer Express Applications-Based Primer Design Software (Perkin-Elmer Applied Biosystems). For bcl-2, primers with the sequences 5'-CATGTGTGTGGAGAGCGTCAA-3' and 5'-GCCGGTTCAGGTACTCAGT CA-3' were used, together with a Taqman probe of sequence 5'-CCTGGTGGACAACATCGCCCTGT-3'. For bcl-xL, primers with the sequences 5'-TCCTTGTCTACGCTTCCACG-3' and 5'-GGTCGCAT TGTGGCCTTT-3' were used together with a Taqman probe of sequence 5'-ACAGTGCCCCGCCGAAGGAGA-3'. The bcl-2 and bcl-xL probes were labeled at the 5' end with the reporter dye 6-carboxy-fluorescein and at the 3' end with the quencher 6-carboxy-tetramethyl-rhodamine. A Basic Local Alignment Search Tool (BLAST) search of the National Center for Biotechnology Information (NCBI) database containing all sequences in the GenBank, European Molecular Biology Laboratory (EMBL) database, and the DNA Database of Japan (DDBJ) revealed no homology of the primer and probe sequences to any other known human genes. Data are presented relative to an untreated control sample chosen as calibrator. The range of a given sample relative to the calibrator was always less than 10 %.

Western Blot Analysis

Lysates from cells treated with oligonucleotides were subjected to western blot analysis as described previously (23). Twenty micrograms of soluble protein per sample was separated by 12% polyacrylamide gel containing sodium dodecyl sulfate and transferred to a polyvinylidene fluoride membrane in a semidry blotting chamber for 1 hour. The blots were blocked in Tris-buffered saline containing 5% bovine serum albumin (BSA) and 5% nonfat dry milk and then incubated overnight at 4 °C with mouse anti-human Bcl-2 monoclonal antibody (Dako Diagnostics AG, Glostrup, Denmark) or rabbit anti-human Bcl-x polyclonal antibody (Transduction Laboratories, Lexington, KY). Actin, stained with a mouse anti-actin monoclonal antibody (ICN Biomedicals, Inc., Aurora, OH), was used as a loading control. For the detection of the primary antibodies, we incubated blots with rabbit anti-mouse or goat anti-rabbit immunoglobulin (Ig) peroxidase conjugates (Sigma Chemical Co., St. Louis, MO), respectively, for 1 hour at room temperature. Antibodies were added in Tris-buffered saline containing 1% BSA, 1% nonfat dry milk, and 0.05% Tween 20 (Sigma Chemical Co.). Visualization of the immunocomplexes was performed by enhanced chemiluminescence with the use of the ECLTM Kit (Amersham Pharmacia Biotech), followed by exposure to x-ray films. Relative protein levels were quantified with the use of Scion software (Scion Corp., Frederick, MD) on scanned films.

Measurement of Cell Growth Inhibition

Effects of oligonucleotides on cell growth were determined as described previously (23) with the use of a colorimetric assay based on the reduction of the tetrazolium salt MTT [i.e., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] by mitochondrial dehydrogenases in viable cells. For each experiment, cells were seeded in 96-well plates and cultured for 24 hours. Oligonucleotides were then added in the form of complexes with Lipofectin up to a total volume of 100 μ L, and cells were incubated at 37 °C. Seventy-two hours after the treatment was started, 10 μ L of MTT reagent (10 mg/mL) was added and allowed to react for 90 minutes at 37 °C before the addition of 100 μ L of solubilization reagent (20% sodium dodecyl sulfate in 50% dimethyl formamide [pH 4.7] and pH-adjusting solution [i.e., 80% acetate and 20% 1 M HCl]). Substrate cleavage was monitored 12 hours later at 570 nm by use of a SPECTRAMax 340 microplate reader and analyzed by use of SOFTmax PRO software (Molecular Devices, Sunnyvale, CA).

Measurement of Cell Death and Apoptosis

Tumor cell death and apoptosis were measured 72 hours after the start of treatment with 600 nM oligonucleotide by flow cytometry with the use of a FACScalibur flow cytometer and the CellQuest software (Becton Dickinson, Mountain View, CA). Cells were incubated with 10 μ g/mL propidium iodide for 5 minutes, and the fraction of cells with increased fluorescence intensity was measured. Cell debris was excluded from the analysis by appropriate light scatter gating. The apoptotic fraction was determined after the cells were made permeable with 50% ethanol for 30 minutes followed by incubation with 5 μ g/mL

RNase A for 30 minutes and incubation with 40 μ g/mL propidium iodide for 10 minutes. After clusters and fragments were excluded (27), cells with sub-G₀/G₁ DNA profiles were identified. In addition, apoptosis was assessed by nuclear staining with Hoechst 33342 dye (Sigma Chemical Co.). For this purpose, cells were pelleted, resuspended, and fixed in 4% paraformaldehyde–0.05% saponin–5 μ g/mL Hoechst 33342 for 15 minutes before cytospin centrifugation (20g for 3 minutes at room temperature). Cells were mounted with Mowiol (Calbiochem, La Jolla, CA), and nuclei were observed by use of a Leica confocal laserscan microscope equipped with SCANware software (Leitz, Wetzlar, Germany). Images were processed with the use of the Imaris software (Bitplane, Zurich, Switzerland).

Determination of Antitumor Activity *In Vivo*

Six- to 8-week-old female CD1 nu/nu (nude) mice were obtained from BRL (Fullinsdorf, Switzerland) and kept under specific-pathogen-free conditions. Animal experiments were performed according to the guidelines of the Veterinary Office of Zurich, Switzerland. Tumor xenografts were raised by subcutaneous injection of 10⁷ MDA-MB-231 or Colo-320 cells into the lateral flanks of the mice under ether anesthesia. Growing tumors were measured with calipers, and the approximate tumor volume was calculated by use of the following formula: volume (mm³) = length $\times$ width²/2. Once tumors had reached an average size of 75 mm³, mice were randomly assigned to treatment groups matched for equal mean tumor volumes. Oligonucleotides were injected intraperitoneally at a dose of 20 mg/kg per day over a period of 3 weeks. MDA-MB-231 tumors were measured after treatment for 0, 8, 13, 18, and 22 days, while Colo-320 tumors were measured after 0, 6, 11, 15, and 21 days.

Detection of Bcl-2 and Bcl-xL Proteins in Tumor Xenografts

After 2 weeks of treatment as described above, mice were killed by cervical dislocation, and their tumors were removed, immersed in Hanks' balanced salt solution, and snap-frozen in liquid nitrogen. One half of each tumor was analyzed by immunohistochemical staining for Bcl-2 and Bcl-x; the other half was lysed and used for western blotting of Bcl-2 and Bcl-xL.

For immunohistochemistry, 5- μ m tissue sections were cut in a cryostat, placed on siliconized glass slides, air-dried, fixed with acetone for 10 minutes, and stored at –70 °C. Bcl-2 was stained by incubating rehydrated sections with primary fluoresceinated mouse monoclonal antibodies against human Bcl-2 (clone 124; Dako Diagnostics AG), followed sequentially by rabbit polyclonal anti-fluorescein isothiocyanate antibodies (Dako Diagnostics AG) and alkaline phosphatase-labeled donkey anti-rabbit Ig antibodies (Jackson Laboratories, West Grove, CA). Bcl-x was stained with the use of polyclonal rabbit anti-Bcl-x antibodies (Transduction Laboratories). Primary rabbit antibodies were revealed by sequential incubation with alkaline phosphatase labeled goat anti-rabbit Ig antibodies and donkey anti-goat Ig antibodies (Jackson Laboratories). Dilutions of the affinity-purified secondary and tertiary antibodies were made in Tris-buffered saline containing 5% normal mouse serum. Alkaline phosphatase was visualized with the use of naphthol AS-BI (6-bromo-2-hydroxy-3-naphtholic acid-2-methoxy anilide) phosphate and new fuchsin as substrate. Endogenous alkaline phosphatase was blocked by levamisole. Color reactions were performed at room temperature for 15 minutes with reagents (Sigma Chemical Co.). Sections were counterstained with hemalum, and coverslips were mounted with glycerol and gelatin.

For the determination of the levels of Bcl-2 and Bcl-xL proteins in tumor lysates, tumors were homogenized with the use of a Polytron PT 3000 (Kinematic AG, Littau, Switzerland), and protein extraction and immunoblotting were performed as described above under the heading entitled "Western Blot Analysis."

Detection of Apoptosis in Tumor Xenografts

For TUNEL (i.e., terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick end labeling) staining, formalin-fixed and paraffin-embedded tumor sections (4 μ m) were deparaffinized and rehydrated by standard procedures. The tissues were rinsed briefly in Tris-buffered saline (i.e., 0.15 M NaCl and 50 mM Tris-HCl [pH 7.4]) and digested with 30 μ g/mL proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) in Tris-buffered saline for 20 minutes at room temperature. After being washed in distilled water for 10 minutes, the sections were equilibrated for 15 minutes in TdT reaction buffer (i.e., 200 mM potassium cacodylate, 2.5 mM CoCl₂, 0.25

Fig. 1. Inhibition of bcl-2 and bcl-xL messenger RNA (mRNA) and protein expression in MDA-MB-231 breast carcinoma cells. **A)** Cells were treated for 20 hours with antisense oligonucleotide 4625 or control oligonucleotides 4626 and 4258 at a concentration of 600 nM. Cells were harvested, and mRNA levels of bcl-xL and bcl-2 were determined by reverse transcription–polymerase chain reaction. β 2-Microglobulin (β 2-MG) was used as a control for RNA quantity and integrity. Bands are shown from one representative experiment of three experiments. **B)** Cells were treated for 20 hours with antisense oligonucleotide 4625 or control oligonucleotides 4626 and 4258 at the concentrations indicated (nM). Cells were harvested, and mRNA levels of bcl-xL (**solid bars**) and bcl-2 (**open bars**) were assessed by reverse transcription followed by real-time PCR quantification. The levels in untreated cells measured under identical experimental conditions were taken as 100%. Data represent the mean and 95% confidence interval ($n = 3$). **C)** Cells were treated for 40 hours with 600 nM antisense oligonucleotide 4625 or control oligonucleotides 4626 or 4258. Cells were harvested, and Bcl-2 and Bcl-xL protein levels were determined by western blotting. Bands from one representative experiment are shown; **numbers** indicate the mean and 95% confidence interval ($n = 3$) given as percentage of the Bcl-2 and Bcl-xL levels in untreated cells. β -Actin was used as a loading control. Bcl-xL migrated at 31 kilodaltons (kd), Bcl-2 at 28 kd, and β -actin at 48 kd.

mg/mL BSA, and 25 mM Tris–HCl [pH 6.6]). The sections were then covered with the TUNEL reagent mix (62.5 U/mL calf thymus TdT [Boehringer Mannheim GmbH] and 3.75 nmol/mL digoxigenin-11-deoxyuridine triphosphate in TdT reaction buffer) and incubated for 1 hour at 37 °C in a water-saturated atmosphere. The sections were washed for 10 minutes with 2 $\times$ standard saline citrate at 40 °C, rinsed with Tris-buffered saline, and preincubated with blocking buffer (2% BSA and 0.3% Triton X-100 in Tris-buffered saline) for 30 minutes at room temperature. For the detection of digoxigenin, sections were treated for 1 hour with alkaline phosphatase-labeled Fab fragments of affinity-purified sheep anti-digoxigenin antibodies (1 U/mL blocking buffer at room temperature). After a further washing step with Tris-buffered saline, the red reaction product was developed under visual control with the use of naphthol AS-BI phosphate and new fuchsin as substrate. Endogenous alkaline phosphatase was blocked by levamisole. After being counterstained with hemalum, coverslips were mounted with glycerol–gelatin. Tissue sections from human tonsils, typically showing apoptotic cells, were used as positive controls.

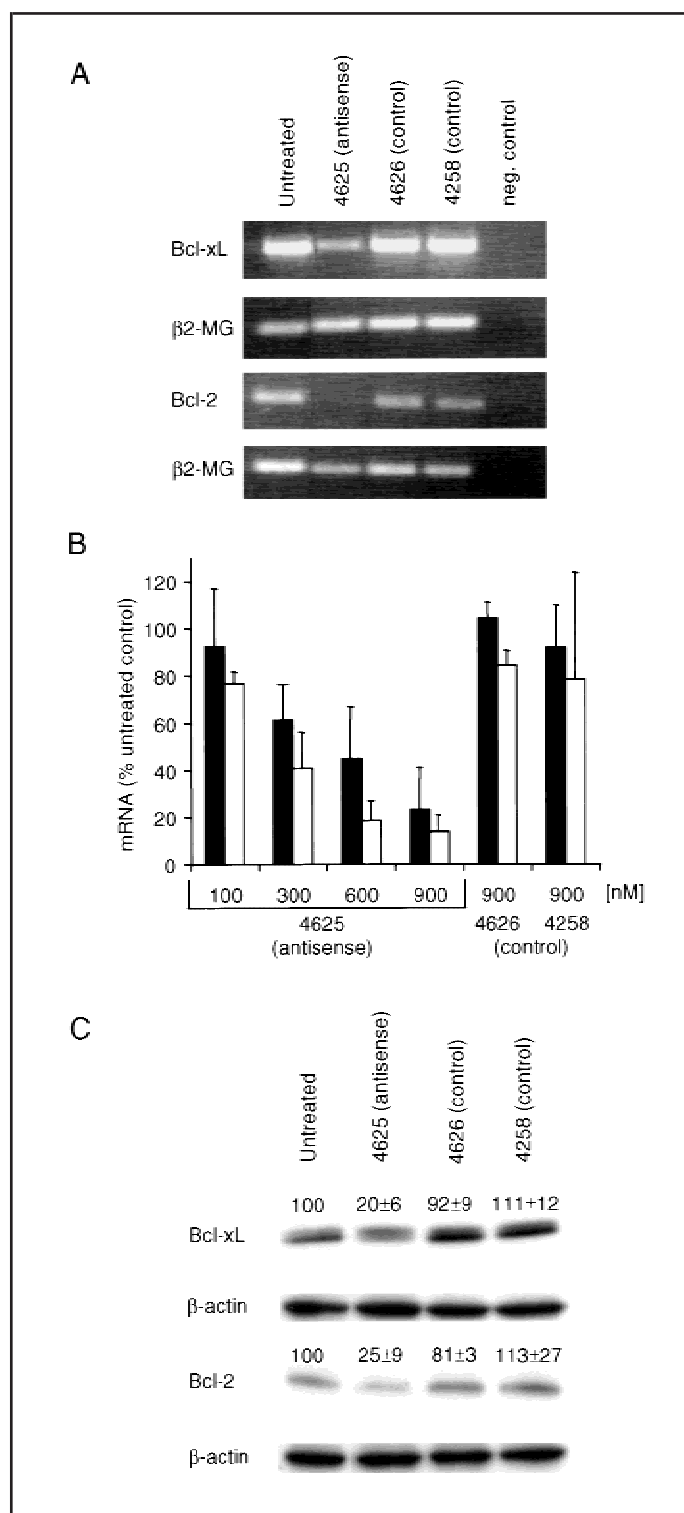
Statistical Analysis

If not stated otherwise, data represent the mean and 95% confidence interval (CI) of at least three independent determinations. *In vitro* growth assays were analyzed with the use of analysis of variance (ANOVA) for repeated measurements with the two within-factors concentration and treatment. Paired *t* tests were used to determine the statistical significance of the antisense effect on cell death compared with the control treatment, *P* values of less than .05 were considered to be statistically significant, and all statistical tests were two-sided. The effect of antisense oligonucleotide 4625 and control oligonucleotide 4626 on tumor growth in mice was analyzed with the use of ANOVA for repeated measurements. Within-factor time and between-factor treatment were compared by use of Bonferroni–Dunn *post-hoc* tests.

RESULTS

Inhibition of bcl-2 and bcl-xL mRNA and Protein Expression by Oligonucleotide 4625

Oligonucleotide 4625 is 100% complementary to the bcl-2 mRNA and has three base mismatches to the bcl-xL mRNA, two of which were modified by 2'-MOE residues. Based on this design, it has the potential to hybridize to both mRNAs and to inhibit the expression of both genes. To demonstrate this bispecific activity, we treated MDA-MB-231 breast carcinoma cells expressing high levels of Bcl-2 and Bcl-xL with oligonucleotides, and we determined bcl-2 and bcl-xL mRNA by reverse transcription–PCR. Samples treated with antisense oligonucleotide 4625 showed markedly reduced levels of both bcl-2 and bcl-xL mRNAs compared with untreated samples or samples



treated with control oligonucleotide (Fig. 1, A). For precise quantification of sequence-specific inhibition of mRNA expression, real-time PCR was performed. Antisense oligonucleotide 4625 reduced bcl-2 and bcl-xL mRNA in a dose-dependent manner, as determined 20 hours after the start of transfection (Fig. 1, B). The maximal inhibition was achieved with 900 nM oligonucleotide 4625 and was 86% (95% CI = 79% to 93%) and 77% (95% CI = 60% to 94%) for bcl-2 and bcl-xL, respectively. The concentrations at which mRNA expression was inhibited by 50% (IC₅₀ values) were 250 nM and 500 nM (data not

shown). The control oligonucleotides 4626 and 4258 had only marginal effects on *bcl-2* and *bcl-xL* mRNA expression, indicating that the bispecific activity of oligonucleotide 4625 was sequence specific and was not related to unspecific gene-regulatory events. On the protein level, 600 nM oligonucleotide 4625 reduced Bcl-2 and Bcl-xL expression to 25% (95% CI = 16% to 34%) and 20% (95% CI = 14% to 26%), respectively, when measured 40 hours after the start of transfection (Fig. 1, C). Again, the control oligonucleotides 4626 and 4258 did not substantially affect Bcl-2 and Bcl-xL protein levels and, in all further experiments, only oligonucleotide 4626 was used as a control. Bcl-xL migrated as a doublet, and both bands were diminished by antisense treatment. Bcl-xS, a pro-apoptotic splice variant of the *bcl-x* gene, was not detectable under these conditions (data not shown).

Inhibition of Tumor Cell Growth by Oligonucleotide 4625 in Lung, Breast, Colorectal, and Prostate Carcinoma Cells and in Malignant Melanoma Cells

Bcl-2 and Bcl-xL are survival factors protecting cells from apoptosis induced by a variety of exogenous and endogenous stimuli. Having demonstrated the bispecific activity of oligonucleotide 4625 on the level of *bcl-2* and *bcl-xL* mRNA and protein, we further examined its ability to inhibit the *in vitro* growth of a series of cell lines derived from lung, breast, colorectal, and prostate carcinomas and from malignant melanoma. As measured in MTT assays 72 hours after the start of transfection, oligonucleotide 4625 inhibited the growth of all cell lines in a dose-dependent manner, with IC₅₀ values ranging from 90 to 730 nM (Fig. 2). At a concentration of 800 nM, the levels of inhibition ranged from 57% for LoVo cells to 93% for Colo-320 cells. With the exception of LoVo cells ($P = .150$), the inhibition of cell growth was statistically significant (P values ranging from .024 to .004) as compared with control oligonucleotide 4626. The nonspecific cytostatic effect of oligonucleotide 4626 was comparatively low. The optimal antisense concentration, which is defined by a maximum difference in cell growth inhibition achieved with oligonucleotides 4625 and 4626, ranged from 400 to 600 nM.

Induction of Tumor Cell Death and Apoptosis by Oligonucleotide 4625 in Lung, Breast, Colorectal, and Prostate Carcinoma Cells and in Malignant Melanoma Cells

To investigate whether cell growth inhibition was due to the induction of cell death and, more specifically, to apoptosis, we analyzed propidium iodide uptake in unfixed cells and DNA content in

permeabilized cells by flow cytometry 72 hours after the start of transfection with 600 nM oligonucleotides. Antisense treatment induced death in all nine cell lines, although at different rates (Fig. 3, A). Uptake of propidium iodide was highest in A-375 cells (77%), LoVo cells (72%), and Colo-320 cells (65%) and less pronounced in SW2 cells (36%) and PC-3 cells (32%). The effect of oligonucleotide 4625 was statistically significantly stronger when compared with that of the control oligonucleotide 4626 (P values between .010 and .001). Cell death was mainly due to the induction of apoptosis (Fig. 3, B and C). After antisense treatment, a large fraction of cells with typical sub-G₀/G₁ DNA content indicative of apoptosis was observed in Colo-320 cells (64%), A-375 cells (61%), and LoVo cells (51%). Cells with fragmented DNA were less frequent in the SW2 (20%) and PC-3 (8%) cell lines. Differences between antisense and control treatment were statistically significant (P values between .001 and .009) except for PC-3 cells ($P = .053$). Further proof that cell death induced by oligonucleotide 4625 treatment mainly occurred by apoptosis was provided by Hoechst staining of nuclei, which revealed intense nuclear fragmentation and condensation (left inset in Fig. 3, C). With the exception of LoVo cells, which showed a strong response to antisense treatment in the cell death assays but were less responsive when examined in MTT assays, these data reflected the results from the growth-inhibition assays mentioned above. Treatment with the control oligonucleotide 4626 decreased the number of viable cells to a much lesser extent.

In Vivo Antitumor Activity of Oligonucleotide 4625 Against Breast and Colorectal Carcinoma Xenografts

To determine whether the potent cell death-inducing effect of antisense oligonucleotide 4625 observed *in vitro* translated into therapeutic efficacy *in vivo*, we investigated its activity in nude mice bearing established subcutaneous MDA-MB-231 breast carcinoma or Colo-320 colorectal carcinoma xenografts. Oligo-

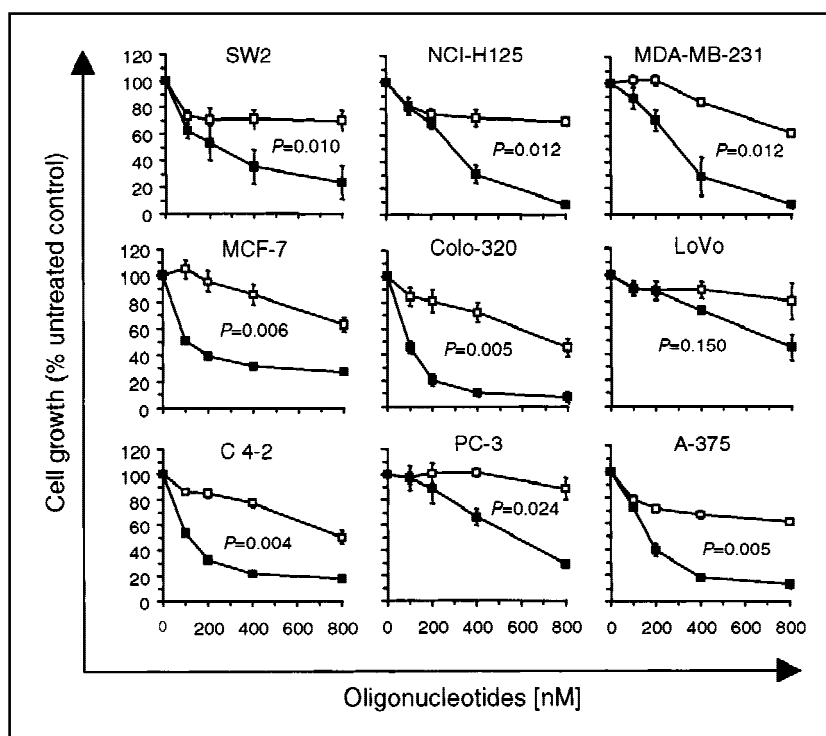


Fig. 2. Inhibition of tumor cell growth in lung carcinoma (SW2 and NCI-H125), breast carcinoma (MDA-MB-231 and MCF-7), colorectal carcinoma (Colo-320 and LoVo), prostate carcinoma (PC-3 and C4-2), and malignant melanoma (A-375) cell lines. Cells were treated for 72 hours with increasing concentrations of antisense oligonucleotide 4625 (solid symbols) or control oligonucleotide 4626 (open symbols), and cell growth was measured in quadruplicate by use of the MTT [i.e., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent. Data represent the mean and 95% confidence interval ($n = 3$). P values were determined with the use of analysis of variance for repeated measurements.

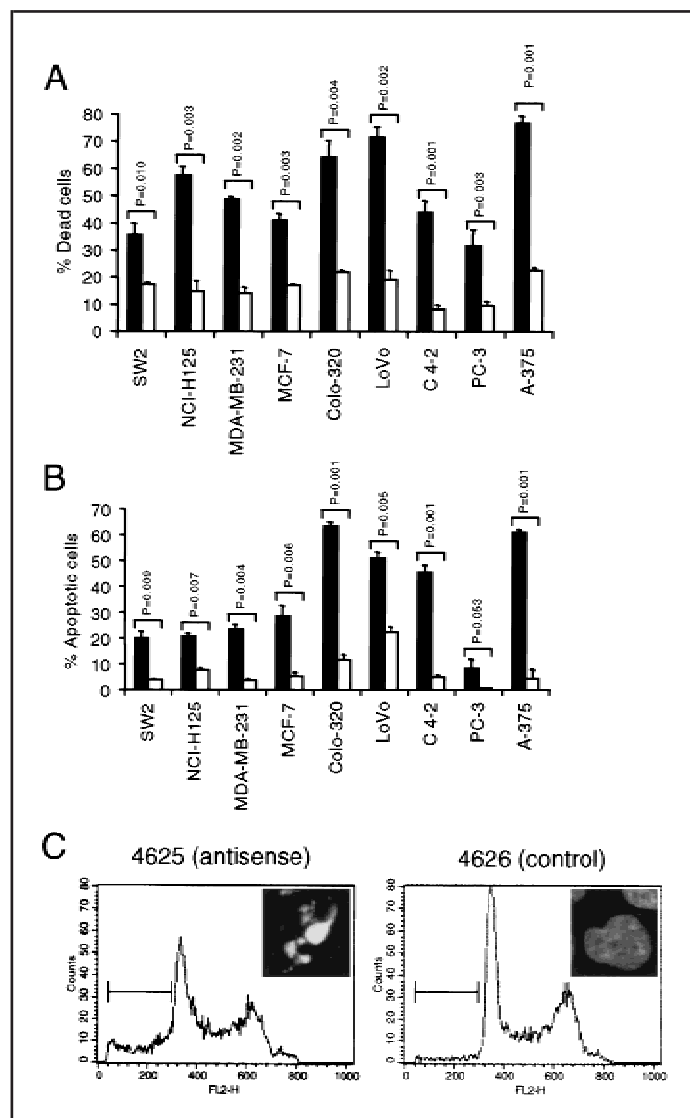


Fig. 3. Induction of tumor cell death and apoptosis in lung carcinoma (SW2 and NCI-H125), breast carcinoma (MDA-MB-231 and MCF-7), colorectal carcinoma (Colo-320 and LoVo), prostate carcinoma (PC-3 and C4-2), and malignant melanoma (A-375) cell lines. Cells were treated for 72 hours with 600 nM antisense oligonucleotide 4625 (solid symbols) or control oligonucleotide 4626 (open symbols). Cell death (A) and apoptosis (B) were assessed on the basis of propidium iodide uptake into unfixed or ethanol-fixed cells, respectively, by use of a flow cytometer. Data represent the mean and 95% confidence interval of three determinations. Paired *t* tests were performed to determine statistical significance. C) Histograms illustrating the appearance of an apoptotic sub- G_0/G_1 cell population upon treatment with antisense oligonucleotide 4625; insets: Hoechst stainings of nuclei confirming the apoptotic morphology of antisense-treated cells. Representative examples of MDA-MB-231 cells from the experiment described in this legend are shown.

nucleotides were injected intraperitoneally at a dose of 20 mg/kg per day over a period of 3 weeks. Antisense treatment statistically significantly inhibited the growth of both breast carcinoma and colorectal carcinoma xenografts. At the end of the 3-week treatment period, oligonucleotide 4625 had diminished the growth of MDA-MB-231 xenografts (Fig. 4, A) by 51% (95% CI = 28% to 74%) ($P = .016$) and of Colo-320 xenografts (Fig. 4, B) by 59% (95% CI = 44% to 74%) ($P < .001$) as compared with control oligonucleotide 4626. There was no statistically significant difference in the growth of untreated and

control oligonucleotide 4626-treated tumors ($P = .65$ and $P = .78$, respectively). No signs of treatment-related toxicity to the animals, such as inflammation, bleeding, hepatomegaly and splenomegaly, or weight loss, were observed over the course of the study.

Inhibition of Bcl-2 and Bcl-xL Protein Expression and Induction of Apoptosis by Oligonucleotide 4625 in Tumor Xenografts

To provide evidence that the antitumor activity of oligonucleotide 4625 was due to its ability to downregulate Bcl-2 and Bcl-xL *in vivo*, we analyzed tumor xenografts treated for 2 weeks as described above by immunohistochemistry. For this purpose, MDA-MB-231 breast carcinoma xenografts were chosen because they express Bcl-2 and Bcl-xL at readily detectable levels but no splice variant Bcl-xS (data not shown). Fig. 5, A–D, shows a representative tumor xenograft in which Bcl-2- and Bcl-x-positive cells were heterogeneously distributed across the tumor sections. Whereas large areas with intense staining for Bcl-2 and Bcl-x were present in control treated tumors, antisense-treated tumors showed markedly less intense staining for both proteins in the majority of the cells, and this finding did not depend on the cell density (Fig. 5, A–D). Tumor sections processed with secondary and tertiary antibodies alone were used as negative controls (Fig. 5, E and F). Fig. 5, G, shows an immunoblot of whole-tumor lysates from representative tumor xenografts confirming the inhibition of target protein expression shown by immunostaining. The lower inhibitory effect on Bcl-xL expression might be due to the presence of mouse stromal and endothelial cells in the tumor lysate and the fact that, in contrast to Bcl-2, available antibodies do not discriminate between human and mouse Bcl-xL.

To determine whether oligonucleotide 4625 induced apoptosis in the tumors, we performed TUNEL staining 1 week after the start of treatment to detect the presence of apoptotic nuclei *in situ*. Although in all tumors few isolated TUNEL-positive nuclei were visible, oligonucleotide 4625 antisense-treated tumors clearly showed more such apoptotic nuclei (Fig. 5, H) than did control oligonucleotide 4626-treated tumors (Fig. 5, I). Thus, as expected from its proposed mechanism of action, antisense oligonucleotide 4625 reduced Bcl-2 and Bcl-xL protein levels and induced apoptosis in the tumor xenografts.

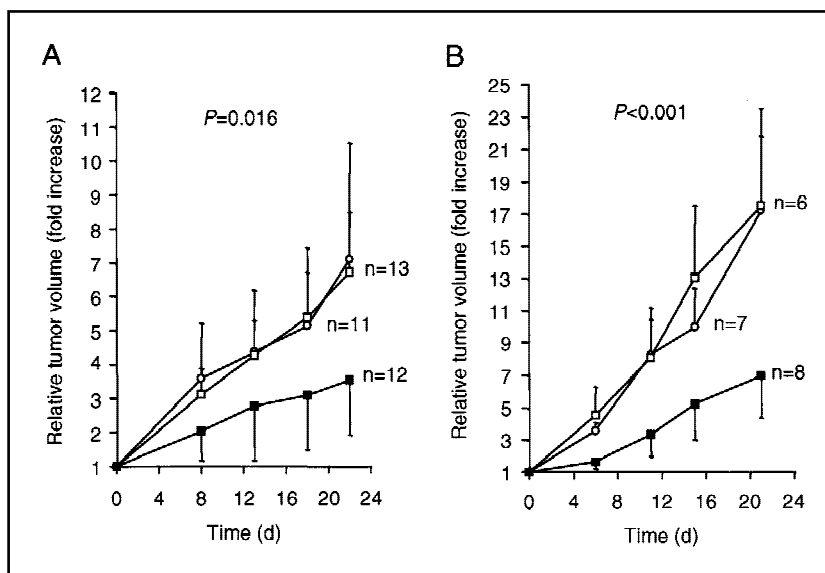
DISCUSSION

The bcl-2 or bcl-xL antisense compounds currently under clinical investigation are specific for either bcl-2 or bcl-xL, a property limiting their therapeutic efficacy in the frequent cases of advanced tumors where both cell death antagonists are expressed. The rationale behind the antisense approach described in our study was the use of an antisense oligonucleotide with the ability to simultaneously downregulate bcl-2 and bcl-xL expression. This property is likely to be of therapeutic benefit and may have a broader applicability in cancer therapy compared with the bcl-2- and bcl-xL-monospecific oligonucleotides described so far. We have recently designed the bcl-2/bcl-xL-bispecific antisense oligonucleotide 4625 and reported its ability to simultaneously inhibit the expression of bcl-2 and bcl-xL and induce apoptosis in small-cell lung carcinoma and lung adenocarcinoma cells (25). Here, we describe the use of this bispecific antisense compound to inhibit the growth and induce apoptosis in tumor cells of diverse histologic origins *in vitro* and *in vivo*. We have

Fig. 4. Growth inhibition of tumor xenografts. Nude mice bearing established, subcutaneously growing MDA-MB-231 breast (A) or Colo-320 colorectal (B) carcinoma xenografts either remained untreated (**open circles**) or were treated with 20 mg/kg per day of antisense oligonucleotide 4625 (**solid squares**) or control oligonucleotide 4626 (**open squares**) administered intraperitoneally over a period of 3 weeks. Tumor size was measured, and the relative increase in tumor volume was calculated at the time points indicated. Data represent the mean and 95% confidence interval (n = number of animals per group). P values were determined with the use of analysis of variance for repeated measurements.

tested nine cell lines derived from lung, breast, colorectal, and prostate carcinomas, representing the four most prevalent and malignant tumors, as well as cells from malignant melanoma. These cell lines express distinct levels of Bcl-2 and Bcl-xL that were representative of the respective tumor types (28–32).

As shown by conventional reverse transcription–PCR and real-time PCR, oligonucleotide 4625 efficiently inhibited bcl-2 and bcl-xL mRNA expression in MDA-MB-231 breast adenocarcinoma cells in a dose-dependent manner. Northern blot analysis yielded identical results for the bcl-xL mRNA, whereas the inhibitory effect of oligonucleotide 4625 on bcl-2 mRNA expression could not be evaluated, since the signal was masked by strong background corresponding to the 28S rRNA (data not shown). As expected from its complementarity to the target sequences (no mismatch to bcl-2 and three mismatches to bcl-xL), the IC_{50} value for inhibiting mRNA expression was higher for bcl-xL than for bcl-2 (500 nM versus 250 nM). However, 900 nM oligonucleotide 4625 reduced both bcl-2 and bcl-xL mRNA levels to a similar extent, suggesting that the 2'-MOE-modified nucleotide building block could indeed largely overcome the three mismatches to bcl-xL to yield a truly bcl-2/bcl-xL-bispecific antisense compound. On the protein level, oligonucleotide 4625 was effective equally on Bcl-2 and Bcl-xL already at a concentration of 600 nM, a finding possibly reflecting differences in the half-lives of these proteins. In all tumor cell lines tested, treatment with 600 nM antisense oligonucleotide 4625 resulted in cell death, which was mainly due to the induction of apoptosis. The quantitative differences in the overall rate of cell death and apoptosis observed in some cell types suggest that cell death did not always occur by classic apoptosis. These differences may also have been due to the fact that, at more advanced stages, the apoptosis process cannot be distinguished from other forms of cell death (27). The colorectal carcinoma cell line Colo-320 and the malignant melanoma cell line A-375 were the most susceptible to antisense treatment, while the SW2 small-cell lung carcinoma cell line and the PC-3 prostate carcinoma cell line were the least susceptible. At present, we cannot exclude cell type-specific differences in oligonucleotide uptake and intracellular distribution (33), but the differential responsiveness of the cell lines to the inhibition of Bcl-2 and Bcl-xL expression suggests differences in the way in which they respond to changes in the life–death rheostat maintained by pro- and anti-apoptotic Bcl-2 family members. We further measured Bcl-2, Bcl-xL, and Bax levels by western blotting in all the cell lines used in this study (data not shown), but we could not establish a positive or negative association between the basal levels of these proteins and the results obtained in the



cell viability and apoptosis assays. In view of the complexity of the apoptosis machinery and the fact that a great number of pro- and anti-apoptotic proteins are engaged in apoptosis regulation, this finding is not surprising. Intraperitoneal injection of oligonucleotide 4625 statistically significantly inhibited the growth of established subcutaneously growing MDA-MB-231 breast and Colo-320 colorectal carcinoma xenografts in nude mice. The injected dose of 20 mg/kg per day of oligonucleotide 4625 was chosen because it proved to be both effective and well tolerated in a study with a similar type of antisense oligonucleotide targeting the C-raf kinase (34). The antitumor effect of oligonucleotide 4625 was associated with a reduction in Bcl-2 and Bcl-xL proteins in the carcinoma xenografts, as demonstrated by immunostaining *in situ* and western blot analysis of whole-tumor lysates. The observation that the overall cell density on the tumor sections was somewhat lower in the antisense-treated than in the control-treated xenografts may be the result of increased apoptotic cell loss and the subsequent replacement of tumor tissue by connective tissue in the antisense-treated tumors. *In situ* TUNEL staining indeed revealed that tumors from mice treated with oligonucleotide 4625 showed clearly more apoptotic cells than control tumors from mice treated with oligonucleotide 4626. GenBank analysis revealed that oligonucleotide 4625 has more than six mismatches to the murine bcl-2 and bcl-xL mRNAs. Thus, despite the promising antitumor effect achieved with our antisense compound, we cannot draw conclusions regarding toxicity related to the reduction in Bcl-2 and Bcl-xL in normal tissues. In recent clinical studies (35,36), however, a bcl-2-monospecific antisense oligonucleotide revealed only minimal side effects, which were attributed mainly to the biochemical properties of the oligonucleotide's phosphorothioate backbone.

In a recent study (37), support was provided for the hypothesis that high-level expression of Bcl-2 and Bcl-xL confers a chemoresistant phenotype on tumor cells. Attempts to compare the expression of Bcl-2 or other anti-apoptotic Bcl-2 family members in tumor cells before treatment and at the time of relapse have demonstrated an apparent increase in Bcl-2 and a survival advantage for cells containing higher Bcl-2 levels (38). The complexity of dealing with a great number of proteins in-

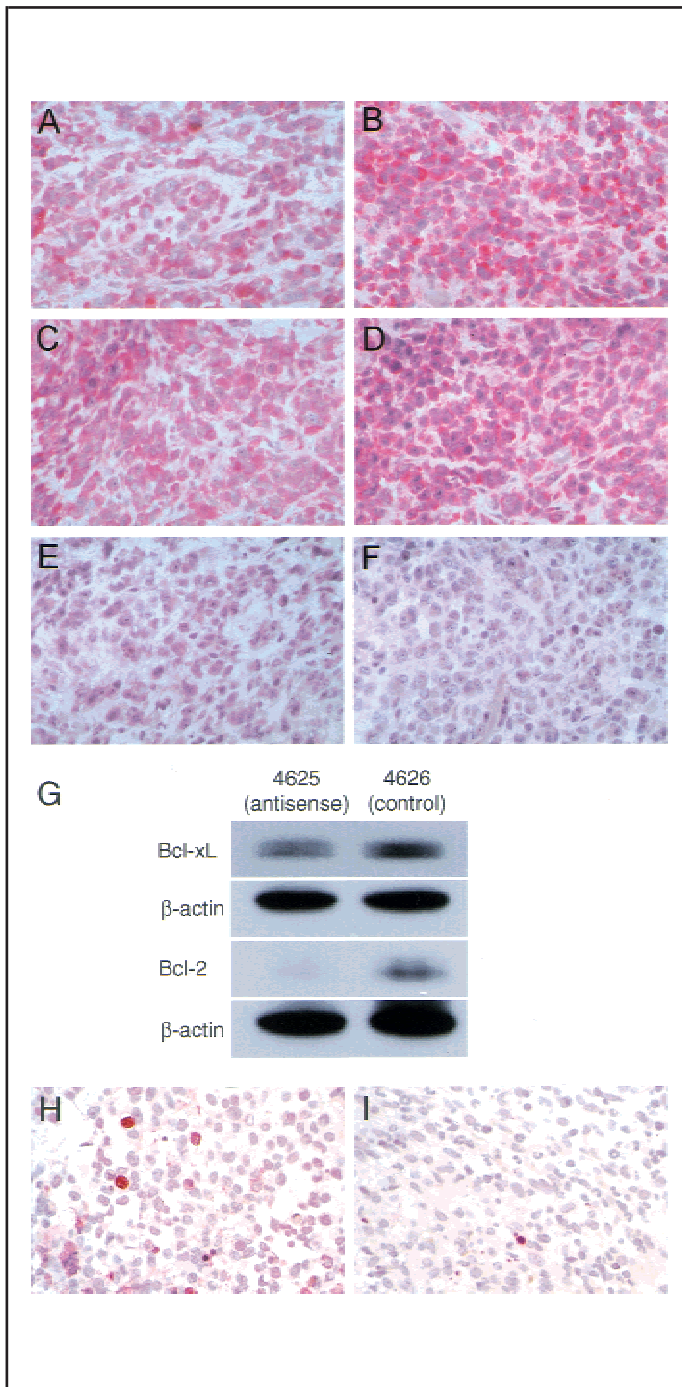


Fig. 5. Inhibition of target protein expression and induction of apoptosis in tumor xenografts. Nude mice bearing established, subcutaneously growing MDA-MB-231 breast carcinoma xenografts received antisense oligonucleotide 4625 (**A**, **C**, **E**, **G**, and **H**) or control oligonucleotide 4626 (**B**, **D**, **F**, **G**, and **I**) administered as described in the legend to Fig. 4. Tumors were dissected 2 weeks after the start of treatment, stained for Bcl-x (**A** and **B**) or Bcl-2 (**C** and **D**) by immunohistochemistry, and counterstained with hemalum. Tumor sections processed with secondary and tertiary antibodies alone were used as negative controls (**E** and **F**). Original magnification $\times 125$. **G**) Immunoblotting of tumor lysates was performed as described in the "Materials and Methods" section to quantify the levels of Bcl-2 and Bcl-xL in antisense-treated and control treated tumors. Bands from one representative tumor per group are shown. Bcl-xL migrated at 31 kilodaltons (kd), Bcl-2 at 28 kd, and β -actin at 48 kd. TUNEL (i.e., terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick end labeling) staining was performed to detect apoptotic cells in antisense oligonucleotide 4625-treated tumors (**H**) or in control oligonucleotide 4626-treated tumors (**I**) 1 week after the start of treatment. Sections from one representative tumor per group are shown. Original magnification $\times 125$.

involved in the apoptosis machinery that serve opposing functions limits the use of Bcl-2 and Bcl-xL as independent prognostic factors and suggests the examination of other proteins, such as the pro-apoptotic Bcl-2 family members. This issue, however, has not always been addressed consistently; probably, for this reason, clinical correlative studies have not uniformly demonstrated an adverse prognostic role for Bcl-2, and some have even shown that overexpression of Bcl-2 in tumors was associated with a favorable prognosis (39–41). Another important issue arises from the fact that, apart from the anti-apoptotic protein Bcl-xL, the bcl-x gene also encodes for pro-apoptotic Bcl-xS by alternative splicing of the bcl-x pre-mRNA (42). Although Bcl-xL seems to be the prevalent form in tumor cells and the relevance of Bcl-xS as a tumor suppressor is rather unclear (43), the potential to target the bcl-xS mRNA in tumor cells could be a fundamental drawback of the bcl-x antisense oligonucleotides described so far. This drawback may not apply to oligonucleotide 4625 for the following reasons: 1) The identified high homology region on the bcl-xL mRNA is localized at a splice junction site of bcl-x, and this sequence occurs neither in the mRNA of Bcl-xS nor in the mRNAs of other pro-apoptotic Bcl-2 family members; and 2) in the bcl-x pre-mRNA, the sequence targeted by oligonucleotide 4625 is interrupted by an intron, excluding the possibility that this oligonucleotide hybridizes to the bcl-x pre-mRNA.

In summary, our results demonstrate that state-of-the-art antisense technology offers a powerful approach to simultaneously target the expression of the two major anti-apoptotic proteins Bcl-2 and Bcl-xL with a single, rationally designed oligonucleotide. The functional advantage of oligonucleotide 4625's bispecificity and its broad activity against tumor cells of diverse histologic origins suggest the use of this antisense compound in cancer therapy.

REFERENCES

- (1) Chao DT, Korsmeyer SJ. Bcl-2 family: regulators of cell death. *Annu Rev Immunol* 1998;16:395–419.
- (2) Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998;281:1309–12.
- (3) Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science* 1998;281:1322–6.
- (4) Simonian PL, Grillot DA, Nunez G. Bcl-2 and Bcl-XL can differentially block chemotherapy-induced cell death. *Blood* 1997;90:1208–16.
- (5) Lee JU, Hosotani R, Wada M, Doi R, Kosiba T, Fujimoto K, et al. Role of Bcl-2 family proteins (Bax, Bcl-2 and Bcl-X) on cellular susceptibility to radiation in pancreatic cancer cells. *Eur J Cancer* 1999;35:1374–80.
- (6) Borner C. Diminished cell proliferation associated with the death-protective activity of Bcl-2. *J Biol Chem* 1996;271:12695–8.
- (7) Bonnotte B, Favre N, Moutet M, Fromentin A, Solary E, Martin M, et al. Bcl-2-mediated inhibition of apoptosis prevents immunogenicity and restores tumorigenicity of spontaneously regressive tumors. *J Immunol* 1998;161:1433–8.
- (8) Groeger AM, Caputi M, Esposito V, De Luca A, Salat A, Murabito M, et al. Bcl-2 protein expression correlates with nodal status in non small cell lung cancer. *Anticancer Res* 1999;19:821–4.
- (9) Holmqvist P, Lundstrom M, Stal O. Apoptosis and Bcl-2 expression in relation to age, tumor characteristics and prognosis in breast cancer. *South-East Sweden Breast Cancer Group. Int J Biol Markers* 1999;14:84–91.
- (10) Olopade OI, Adeyanju MO, Safa AR, Hagos F, Mick R, Thompson CB, et al. Overexpression of BCL-x protein in primary breast cancer is associated with high tumor grade and nodal metastases. *Cancer J Sci Am* 1997;3:230–7.
- (11) Krajewska M, Moss SF, Krajewski S, Song K, Holt PR, Reed JC. Elevated expression of Bcl-X and reduced Bak in primary colorectal adenocarcinomas. *Cancer Res* 1996;56:2422–7.

- (12) Krajewska M, Krajewski S, Epstein JI, Shabaik A, Sauvageot J, Song K, et al. Immunohistochemical analysis of bcl-2, bax, bcl-x, and mcl-1 expression in prostate cancers. *Am J Pathol* 1996;148:1567–76.
- (13) Scherr DS, Vaughan ED Jr, Wei J, Chung M, Felsen D, Allbright R, et al. BCL-2 and p53 expression in clinically localized prostate cancer predicts response to external beam radiotherapy. *J Urol* 1999;162:12–6.
- (14) Grover R, Wilson GD. Bcl-2 expression in malignant melanoma and its prognostic significance. *Eur J Surg Oncol* 1996;22:347–9.
- (15) Han Z, Chatterjee D, Early J, Pantazis P, Hendrickson EA, Wyche JH. Isolation and characterization of an apoptosis-resistant variant of human leukemia HL-60 cells that has switched expression from Bcl-2 to Bcl-xL. *Cancer Res* 1996;56:1621–8.
- (16) Arriola EL, Rodriguez-Lopez AM, Hickman JA, Chresta CM. Bcl-2 overexpression results in reciprocal downregulation of Bcl-X(L) and sensitizes human testicular germ cell tumours to chemotherapy-induced apoptosis. *Oncogene* 1999;18:1457–64.
- (17) Crooke ST. Antisense therapeutics. *Biotechnol Genet Eng Rev* 1998;15: 121–57.
- (18) Monia BP, Lesnik EA, Gonzalez C, Lima WF, McGee D, Guinasso CJ, et al. Evaluation of 2'-modified oligonucleotides containing 2'-deoxy gaps as antisense inhibitors of gene expression. *J Biol Chem* 1993;268: 14514–22.
- (19) Cotter FE, Johnson P, Hall P, Pocock C, al Mahdi N, Cowell JK, et al. Antisense oligonucleotides suppress B-cell lymphoma growth in a SCID-hu mouse model. *Oncogene* 1994;9:3049–55.
- (20) Jansen B, Schlagbauer-Wadl H, Brown BD, Bryan RN, van Elsland A, Muller M, et al. bcl-2 antisense therapy chemosensitizes human melanoma in SCID mice. *Nat Med* 1998;4:232–4.
- (21) Koty PP, Zhang H, Levitt ML. Antisense bcl-2 treatment increases programmed cell death in non-small cell lung cancer cell lines. *Lung Cancer* 1999;23:115–27.
- (22) Zangemeister-Wittke U, Schenker T, Luedke GH, Stahel RA. Synergistic cytotoxicity of bcl-2 antisense oligodeoxynucleotides and etoposide, doxorubicin and cisplatin on small-cell lung cancer cell lines. *Br J Cancer* 1998;78:1035–42.
- (23) Leech SH, Olie RA, Gautschi O, Simões-Wust AP, Tschopp S, Haner R, et al. Induction of apoptosis in lung-cancer cells following bcl-xL anti-sense treatment. *Int J Cancer* 2000;86:570–6.
- (24) Simões-Wust AP, Olie RA, Gautschi O, Leech SH, Haner R, Hall J, et al. Bcl-xL antisense treatment induces apoptosis in breast carcinoma cells. *Int J Cancer* 2000;87:582–90.
- (25) Zangemeister-Wittke U, Leech SH, Olie RA, Simões-Wust AP, Gautschi O, Luedke GH, et al. A novel bispecific antisense oligonucleotide inhibiting both bcl-2 and bcl-xL expression efficiently induces apoptosis in tumor cells. *Clin Cancer Res* 2000;6:2547–55.
- (26) De Mesmaeker A, Haener R, Martin P, Moser HE. Antisense oligonucleotides. *Acc Chem Res* 1995;28:366–75.
- (27) Darzynkiewicz Z, Juan G, Li X, Gorczyca W, Murakami T, Traganos F. Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). *Cytometry* 1997;27:1–20.
- (28) Reeve JG, Xiong J, Morgan J, Bleehe NM. Expression of apoptosis-regulatory genes in lung tumour cell lines: relationship to p53 expression and relevance to acquired drug resistance. *Br J Cancer* 1996;73:1193–200.
- (29) Nieves-Neira W, Pommier Y. Apoptotic response to camptothecin and 7-hydroxystaurosporine (UCN-01) in the 8 human breast cancer cell lines of the NCI Anticancer Drug Screen: multifactorial relationships with topoisomerase I, protein kinase C, Bcl-2, p53, MDM-2 and caspase pathways. *Int J Cancer* 1999;82:396–404.
- (30) Nita ME, Nagawa H, Tominaga O, Tsuno N, Fujii S, Sasaki S, et al. 5-Fluorouracil induces apoptosis in human colon cancer cell lines with modulation of Bcl-2 family proteins. *Br J Cancer* 1998;78:986–92.
- (31) Rokhlin OW, Bishop GA, Hostager BS, Waldschmidt TJ, Sidorenko SP, Pavloff N, et al. Fas-mediated apoptosis in human prostatic carcinoma cell lines. *Cancer Res* 1997;57:1758–68.
- (32) Selzer E, Schlagbauer-Wadl H, Okamoto I, Pehamberger H, Potter R, Jansen B. Expression of Bcl-2 family members in human melanocytes, in melanoma metastases and in melanoma cell lines. *Melanoma Res* 1998;8: 197–203.
- (33) Krieg AM, Gmelig-Meyling F, Gourley MF, Kisch WJ, Chrisey LA, Steinberg AD. Uptake of oligodeoxyribonucleotides by lymphoid cells is heterogeneous and inducible. *Antisense Res Dev* 1991;1:161–71.
- (34) Monia BP. Anti-tumor activity of C-raf antisense—correction [letter]. *Nat Med* 1999;5:127.
- (35) Webb A, Cunningham D, Cotter F, Clarke PA, Di Stefano F, Ross P, et al. BCL-2 antisense therapy in patients with non-Hodgkin lymphoma. *Lancet* 1997;349:1137–41.
- (36) Jansen B, Wachek V, Heere-Ress E, Schlagbauer-Wadl H, Hoeller C, Lucas T, et al. Chemosensitisation of malignant melanoma by BCL2 antisense therapy. *Lancet* 2000;356:1728–33.
- (37) Strasser AM, Huang DC, Vaux DL. The role of the bcl-2/ced-9 gene family in cancer and general implications of defects in cell death control for tumorigenesis and resistance to chemotherapy. *Biochim Biophys Acta* 1997;1333:F151–78.
- (38) Krajewski S, Chatten J, Hanada M, Reed JC. Immunohistochemical analysis of the Bcl-2 oncoprotein in human neuroblastomas. Comparisons with tumor cell differentiation and N-Myc protein. *Lab Invest* 1995;72:42–54.
- (39) Pezzella F, Turley H, Kuzu I, Tungekar MF, Dunnill MS, Pierce CB, et al. bcl-2 protein in non-small-cell lung carcinoma. *N Engl J Med* 1993;329: 690–4.
- (40) Hellemans P, van Dam PA, Weyler J, van Oosterom AT, Buytaert P, Van Marck E. Prognostic value of bcl-2 expression in invasive breast cancer. *Br J Cancer* 1995;72:354–60.
- (41) Baretton GB, Diebold J, Christoforis G, Vogt M, Muller C, Dopfer K, et al. Apoptosis and immunohistochemical bcl-2 expression in colorectal adenomas and carcinomas. Aspects of carcinogenesis and prognostic significance. *Cancer* 1996;77:255–64.
- (42) Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, et al. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* 1993;74:597–608.
- (43) Fulda S, Friesen C, Los M, Scaffidi C, Mier W, Benedict M, et al. Betulinic acid triggers CD95 (APO-1/Fas)- and p53-independent apoptosis via activation of caspases in neuroectodermal tumors. *Cancer Res* 1997;57: 4956–64.

NOTES

O. Gautschi and S. Tschopp contributed equally to this work.

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Specificity of antisense oligonucleotides *in vivo*

(*Xenopus* oocytes)

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ABSTRACT Antisense oligonucleotides are widely used as inhibitors of gene expression in cultured cells and have been proposed as potential therapeutic agents, but it is not known to what extent they are specific for their intended target RNAs. Statistical considerations indicate that if oligonucleotides can form hybrids with mRNA molecules *in vivo* by means of short or imperfect regions of complementarity, then the specificity of oligonucleotides as antisense reagents will be greatly compromised. We have used *Xenopus* oocytes as a model system in which to investigate the potential specificity of antisense oligonucleotides *in vivo*. We injected perfect and partially matched antisense oligonucleotides into oocytes and measured the resulting degradation of the target RNA in each case. On the basis of the extent to which antisense oligonucleotides can cause cleavage of RNAs at imperfectly matched target sites, we conclude that in this system it is probably not possible to obtain specific cleavage of an intended target RNA without also causing at least the partial destruction of many nontargeted RNAs.

Many studies have used antisense oligodeoxynucleotides to inhibit gene expression in cultured cells (1). These studies have shown that antisense oligomers targeted to cellular or viral RNA sequences can produce biological effects, and in some cases these effects have been correlated with reduced levels of specific RNAs or proteins. On the basis of these studies, antisense oligomers have been widely proposed as potential therapeutic agents, which could in principle be targeted against any known cellular or viral RNA sequence. To be effective as therapeutic agents, however, they must block expression of their intended target genes with a high degree of specificity, without producing unwanted side effects. Although *in vitro* studies have clearly established that antisense oligomers can inhibit target genes without producing gross toxic effects on cultured cells, there have so far been few studies on their effectiveness in whole organisms. Thus, the therapeutic value of antisense oligomers has yet to be established.

In many cases antisense oligomers cause the cleavage of their target RNAs by RNase H, and so one potential source of toxic side effects is the fortuitous hybridization and cleavage of RNAs other than the intended target RNA. Two lines of evidence have suggested that antisense oligomers are generally specific for their intended target sequences, although both may be questioned. First, the shortest sequence that is likely to be unique within the mRNA pool is 13 bases, and so it has been argued that an antisense oligomer of 13 or more bases is likely to be specific for its intended target. The argument assumes that only a perfect match between the antisense oligomer and a target RNA will lead to hybridization, but this assumption may be incorrect. It has recently been pointed out on theoretical grounds (2) that problems of specificity may be anticipated with both ribozymes and antisense oligomers, since both recognize and cleave their target RNAs by means

of short stretches of hybridization; as the hybridizing sequence is made longer, the dissociation of mismatched hybrids is expected to become slower, and so the rate of cleavage for these mismatched RNAs will be increased relative to that of the intended target species. It is difficult to predict on theoretical grounds the degree to which antisense oligomers will cause degradation of partially matched RNAs *in vivo*, since this will depend on the ability of RNase H to recognize mismatched duplexes, and on hybrid stability, oligomer concentration, and relative kinetics of hybrid formation and oligomer degradation within the relevant cellular compartment(s). The second argument for the specificity of antisense oligomers is that they can produce biological effects on cultured cells that are specific by the criterion that several different antisense oligomers produce the same effect, while control oligomers do not. In some of the most convincing studies, the effect has been reversed by replacing the depleted RNA species (3, 4), demonstrating that the antisense oligomers do not produce detectable side effects in these assays. These results are encouraging, but it is difficult to extrapolate to the *in vivo* situation, since many genes that are not required for viability in culture may nevertheless be required for more subtle functions *in vivo*. Therefore, determining the extent of RNA degradation by partially matched antisense oligomers requires a direct experimental test.

We have been interested in using antisense oligomers to inactivate maternal mRNAs during *Xenopus* development, both to determine the functions of these RNAs and, more generally, to study the effectiveness of oligomers as antisense reagents (5). *Xenopus* oocytes and embryos contain high levels of RNase H, and therefore injection of antisense oligomer causes cleavage of the target RNA (6–9). However, we (5) and others (10, 11) have observed that the dose of antisense oligomer required to destroy a target RNA is frequently toxic to embryos and oocytes, and we suspected that some of this toxicity might arise from the destruction of other RNA species with fortuitous partial matches to the injected oligomer. In the present study, we have estimated the number of RNA species likely to be complementary to a given oligomer as a function of its length and of the degree of mismatching permitted. We have injected partially matched antisense oligomers into *Xenopus* oocytes to determine whether the destruction of partially matched RNAs is likely to be significant in this system. Given the degree to which mismatched antisense oligomers can still cause degradation of their target mRNAs, we conclude that any antisense oligomer sequence is likely to cause at least partial degradation of many RNA species in *Xenopus* oocytes.

MATERIALS AND METHODS

Oligodeoxynucleotide Preparation. Oligonucleotides were synthesized on an Applied Biosystems 391 synthesizer. After

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deprotection, oligomers were dissolved in water, extracted with phenol/chloroform (1:1, vol/vol), precipitated with ethanol, and redissolved in water. Concentrations were determined by assuming 1 A₂₆₀ unit = 33 µg.

Oocyte Treatment. *Xenopus laevis* were purchased from Xenopus I (Ann Arbor, MI), and stage V–VI oocytes were obtained and cultured as described previously (14). For experiments with fibronectin antisense oligomers and for protein labeling experiments, oocytes were defolliculated with collagenase to eliminate potential background from surrounding follicle cells. Oligomers were injected into oocytes in 20–40 nl.

RNA Extraction and Northern Blot Analysis. For each data point, 4–10 oocytes were pooled. RNA was extracted by the SDS/proteinase K method (ref. 15, pp. 7.16–7.17), fractionated on agarose/formaldehyde gels, and blotted onto Gene-Screen membranes (New England Nuclear). Membranes were probed with ³²P-labeled antisense RNA probes as described (ref. 15, pp. 10.34–10.35). The fibronectin probe was transcribed from a 3' cDNA fragment (12), and the Vg1 probe was transcribed from a full-length cDNA (13). To confirm the identity of the 5' Vg1 cleavage fragment obtained with V10/13, blots were re probed with either a 5' probe (positions 766–1123) or a 3' probe (positions 1738–2378). Hybridization signals were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). To estimate the sizes of RNA bands relative to known standards, autoradiographs were analyzed with MacVector software (IBI).

RESULTS

We began by calculating how many RNA species in a *Xenopus* oocyte are likely to be complementary to any given oligomer as a function of its length, and how this number will vary as different degrees of mismatching are permitted. Our estimates are based on the assumption that an oocyte, like a typical higher eukaryotic cell, contains about 10⁴ different mRNA species, of average length 2 kilobases (kb), giving a total RNA sequence complexity of about 2 × 10⁷ bases (16). For simplicity, we treat the RNA population as a random and equal mixture of the four nucleotides A, C, G, and U. These assumptions are only approximate, but although real cells may diverge from this model [for instance, the nucleotide composition of mRNA varies slightly between species (17)], these divergences are not likely to greatly alter the validity of our conclusions (see Discussion).

The expected number of occurrences for a given sequence of *N* bases within the RNA pool is equal to complexity/4^{*N*}. Therefore, the shortest sequence that is likely to be unique within an RNA pool of 2 × 10⁷ bases is 13 bases, and it is often assumed on the basis of this calculation that an oligomer of 13 or more bases will be specific for its intended target RNA. This assumption may, however, be incorrect, if oligomers shorter than 13 bases can form hybrids *in vivo*. In fact, antisense effects in *Xenopus* have been reported with as few as 10 bases (10). Moreover, increasing the length of an antisense oligomer beyond the minimum length that can hybridize would be expected to decrease rather than increase its specificity, since a longer oligomer will contain more internal sequences of sufficient length to hybridize and degrade RNA. For example, a 20-mer contains 9 different consecutive 12-mers, and since any given 12-mer is expected to occur about 1.2 times in the RNA pool, a 20-mer is expected to match about 11 different sites of 12 consecutive bases. Table 1 shows the expected numbers of complementary sites for oligomers of different lengths, allowing for different minimum lengths of complementarity.

Mismatched bases at internal positions are more destabilizing than mismatched flanking sequences, but some degree of internal mismatching may still be compatible with hybridization. If internal mismatches are permitted, the number of sites complementary to a given oligomer is increased. Table

Table 1. Expected number of fortuitous complementary sites if consecutive perfect matches are required

Length of oligomer (<i>L</i>)	Number of complementary sites				
	<i>N</i> = 8	<i>N</i> = 10	<i>N</i> = 12	<i>N</i> = 14	<i>N</i> = 16
10	916	19	—	—	—
15	2441	114	5	0.15	—
20	3967	210	11	0.5	<0.1
25	5493	305	17	0.9	<0.1

The expected number of sites is given by $0.25^N \times (L - N + 1) \times C$, where *L* is the length of the oligomer, *N* is the length of complementarity required for hybridization, and *C* is the complexity of the RNA pool (assumed to be 2 × 10⁷ bases in our calculations).

2 shows how the number of expected sites varies with the length of the oligomer and the degree of mismatching that is permitted. It is striking that a small increase in the number of permitted mismatches produces a dramatic increase in the number of complementary sites.

To test our assumption that RNA sequences can be treated as a random mixture of the four nucleotides, we searched the GenBank data base (vertebrate sequences only) with three randomly selected 10-mer sequences. This section of the data base has a total complexity of about 4 × 10⁷ bases (counting both strands), so a given 10-mer is expected to occur on average about 38 times. The actual occurrences for the three oligomers were 64, 22, and 61, suggesting that our assumption of randomness is at least approximately correct.

These theoretical considerations suggest that there will be intrinsic limits to the specificity of antisense oligomers. In practice, specificity will depend on the rate of cleavage of the intended target species relative to nontarget RNAs, and this in turn will depend on intracellular oligomer concentration, kinetics of hybrid formation and dissociation, and relative rates of cleavage of perfect and partially matched duplexes by RNase H (see ref. 2 for a more quantitative treatment). These factors are difficult to predict, and therefore we sought to determine whether the effect of mismatches is likely to be of practical importance, by asking whether antisense oligomers with partial mismatches can cause degradation of target mRNAs within *Xenopus* oocytes.

Initially, we asked whether antisense-mediated RNA cleavage can be caused by oligomers containing an internal stretch of exact complementarity flanked by unrelated sequences. For a target mRNA, we selected fibronectin, a moderately abundant maternal transcript of 8.2 kb (12). We chose a target site within the fibronectin mRNA that had previously been shown to be accessible to antisense oligonucleotides; this site is within the EIIIA exon, which is present in over 90% of maternal fibronectin transcripts (12). Three different antisense 25-mers were made against this site.

Table 2. Expected number of fortuitous complementary sites if mismatches are permitted at any position

Length of oligomer (<i>L</i>)	Number of complementary sites				
	<i>M</i> = 0	<i>M</i> = 1	<i>M</i> = 2	<i>M</i> = 3	<i>M</i> = 4
10	19	572	>10 ³	>10 ⁴	>10 ⁵
11	5	157	>10 ³	>10 ⁴	>10 ⁵
12	1	43	708	>10 ³	>10 ⁴
13	0.3	12	209	>10 ³	>10 ⁴
14	<0.1	3	61	732	>10 ³
15	<0.1	1	18	229	>10 ³
20	<0.1	<0.1	<0.1	0.6	7

The expected number of sites is given by $3^M/4^L \times L!/[(L - M)! \times M!] \times C$, where *L* is the length of the oligomer, *M* is the number of mismatches permitted, and *C* is the complexity of the RNA pool (assumed to be 2 × 10⁷ bases in our calculations). The term $L!/[(L - M)! \times M!]$ denotes the number of ways that *M* mismatches can be placed along an oligomer of length *L*.

F25/25: 5'-CAGGACTTGAGTAGGTGACCCCTGTA
 F17/25: 5'-CGAGCATTTGAGTAGGTGACCCCTGAT
 F14/25: 5'-CGAGCATTTGAGTAGGTGACCTCGAT
 V10/13: 5'-TGTAACCAACCCG
 V13/13: 5'-TGCAATCACCAG
 V12/13: 5'-TGCAATCACTCAG
 V17: 5'-GTCTCAGCCACACCATA
 C1: 5'-ATCCGGCTCGAACTGACCG
 C2: 5'-NNNNNNNNNNNNNNNNNN

FIG. 1. Oligomers used in this study. Regions of complementarity to intended target sequences are underlined. Numbering is according to ref. 12 for fibronectin mRNA and ref. 13 for Vg1 mRNA. F25/25 is a 25-mer with perfect complementarity to fibronectin mRNA (positions 5355–5379). F17/25 is a 25-mer with 17 bases of complementarity to the same region as F25/25. F14/25 is a 25-mer with 14 bases of complementarity to the same region. Note that the non-complementary flanking regions of F17/25 and F14/25 are randomly selected, and so by chance some bases match the target site. V10/13 is a 13-mer with 10 consecutive bases complementary to Vg1 (positions 1790–1799). V13/13 is a 13-mer with perfect complementarity to Vg1 (positions 852–864). V12/13 is identical to V13/13 except for a single internal mismatch. V17 is a 17-mer complementary to Vg1 (positions 27–43). C1 is a control 20-mer with no known complementarity to any sequence. C2 is a 17-mer of random composition, with an equal mixture of all four bases at each position.

One was a perfectly complementary sequence, while the other two contained 17 or 14 consecutive bases of complementarity flanked by randomly selected sequences on either side (Fig. 1). As controls, we used two oligomers of unrelated sequence and one random 17-mer consisting of a mixture of all four bases at each position. Each oligomer was injected into oocytes at a dose of 50 ng, since this dose is generally sufficient to result in degradation of the majority of a target mRNA species. After 6 hr at room temperature (about $22 \pm 2^\circ\text{C}$), RNA was extracted and analyzed by Northern blots for fibronectin and control transcripts.

As shown in Fig. 2A, fibronectin mRNA is degraded by all three antisense oligomers; in this experiment, the 25/25-mer (25 out of 25 exact matches) produced 79% degradation, while the 17/25-mer and the 14/25-mer produced 32% and 37% degradation, respectively. An unrelated control oligomer produced 13% degradation. A control mRNA (Vg1) was not substantially affected ($\leq 13\%$ degradation) by any of these oligomers. The partially matched oligomers cleave fibronectin mRNA at the same site as the perfectly matched 25-mer, since a cleavage fragment of the expected size is seen with all three antisense oligomers, whereas no discrete fragments are seen with the control oligomers. Four independent experiments gave similar results, as shown in Fig. 2B. Some control oligomers also produced nonspecific degradation of fibronectin mRNA, but this can be distinguished from antisense cleavage at the target site by the absence of the specific cleavage fragment, which was seen in all cases with the antisense oligomers.

Next, we asked whether antisense cleavage could also be produced by a shorter complementary stretch. For this experiment, we used as a target Vg1 mRNA, a moderately abundant maternal transcript of 2.4 kb, which is localized at the vegetal cortex in late-stage oocytes (18). We made a 13-mer with 10 consecutive bases of complementarity (Fig. 1) and injected this oligomer into oocytes at doses of 10 and 50 ng. After at least 6 hr at room temperature, RNA was extracted and analyzed by Northern blotting for Vg1 and fibronectin (control) transcripts. As shown in Fig. 3A (lanes 6 and 7; cf. lane 1), Vg1 was cleaved by injection of the antisense oligomer at both doses (45% and 63% degradation, respectively), while fibronectin RNA was unaffected ($\leq 15\%$

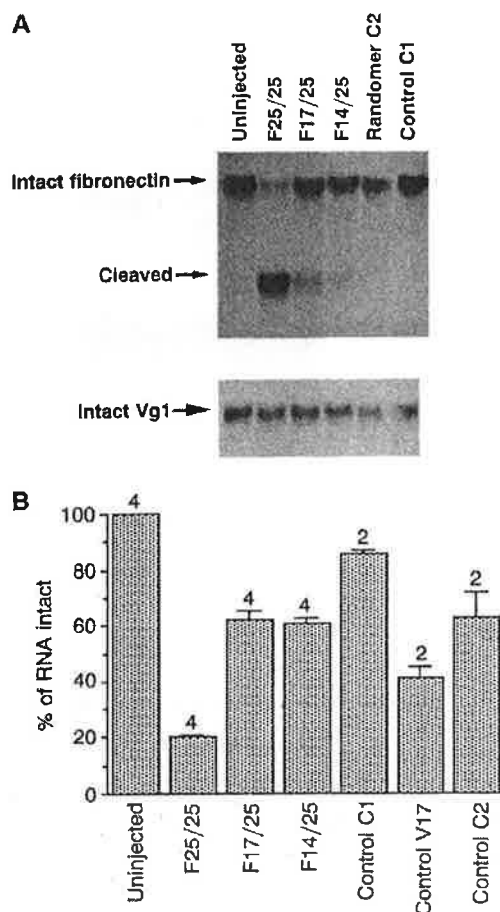


FIG. 2. Cleavage of fibronectin mRNA produced by perfect and partially matched oligomers. Oligomers were injected into oocytes at doses of 50 ng, and fibronectin and Vg1 transcripts were detected by Northern blotting and quantified by using a PhosphorImager. (A) Northern blot of RNA from uninjected oocytes and oocytes injected with the indicated oligomers. The upper gel was probed for fibronectin mRNA. The intact fibronectin transcript is about 8.2 kb. A lower band is visible in the three antisense-injected samples, which corresponds to the 3' cleavage fragment, since it runs at about the correct size (2.8 kb) and reacts with a 3' probe. The lower gel shows the same blot probed for Vg1 mRNA, which is not substantially affected by any of the oligomers. (B) Combined data from four experiments. For each data point, 4–10 oocytes were injected with oligomer as indicated. Oocytes were pooled for RNA extraction, and intact fibronectin RNA was detected by Northern blotting and quantified with a PhosphorImager. For each experiment, results are expressed as percent intact fibronectin mRNA relative to uninjected control oocytes from the same experiment (which are thus 100% by definition). Each bar represents the mean $\pm$ SEM of four or two independent experiments as indicated.

degradation). Three independent experiments gave similar results, as shown in Fig. 3B. In all three experiments a cleavage product of the same size was observed; this band corresponds to the predicted 5' cleavage fragment, since it runs at the expected size and hybridization is detected with a 5' but not with a 3' Vg1 probe (data not shown). We conclude from these experiments that as few as 10 consecutive bases of complementarity are sufficient to hybridize and produce at least some cleavage of a target RNA, and that this is not prevented by mismatched flanking sequences.

We also asked whether antisense cleavage could be produced by oligomers with internal mismatches. A target site was chosen within the Vg1 mRNA that we knew to be accessible from previous experiments (5), and we made two

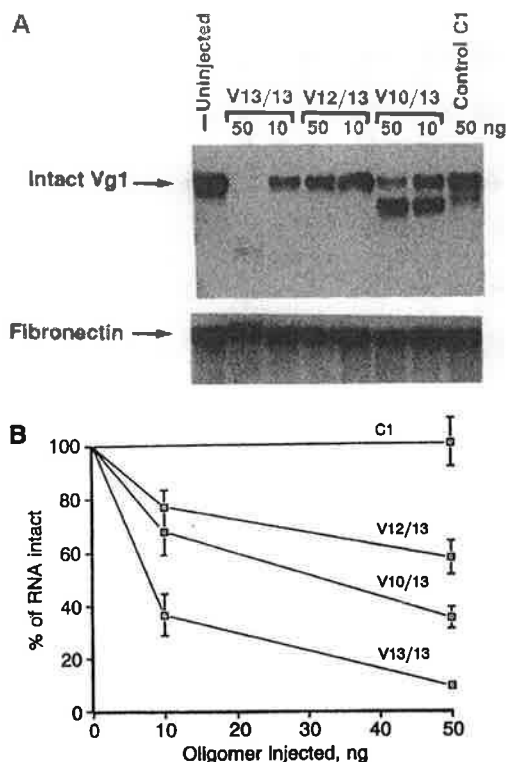


FIG. 3. Cleavage of Vg1 mRNA by perfect and internally mismatched oligomers. Oocytes were injected with 10 or 50 ng of oligomer as indicated, and Vg1 and fibronectin transcripts were detected by Northern blot analysis. (A) Northern blot of RNA from uninjected oocytes and oocytes injected with oligomers V13/13, V12/13, V10/13, or C1 (control) at the doses indicated. The upper gel was probed for Vg1 mRNA; arrow indicates position of intact Vg1 transcript (2.4 kb). The cleavage fragment seen with V13/13 and V12/13 runs at a size close to that predicted for the 5' fragment (0.85 kb). The cleavage fragment seen with V10/13 corresponds to the 5' fragment, since it runs at close to the predicted size (1.8 kb), and it is detected with a 5' but not with a 3' probe (data not shown). The lower gel shows the same blot probed for fibronectin mRNA, which is not significantly affected by any of the oligomers. (B) Combined data from three experiments. For each data point, 4–10 oocytes were injected as indicated. Oocytes were pooled for RNA extraction, and intact Vg1 mRNA was detected by Northern blotting and quantified with a PhosphorImager. For each experiment, results are expressed as percent intact RNA relative to uninjected control oocytes in the same experiment. Each point represents mean $\pm$ SEM of three independent experiments.

different antisense 13-mers to this site, one perfectly complementary, and one with a single mismatch about one third of the way along the oligomer (Fig. 1). Antisense oligomers were injected into oocytes at doses of 10 and 50 ng. We also used a control oligomer (a 20-mer of unrelated sequence) at a dose of 50 ng. As shown in Fig. 3A (lanes 1–5), both the 13/13-mer and the 12/13-mer degraded the Vg1 mRNA in a dose-dependent way, although the 13/13-mer was more effective. Three separate experiments yielded similar results, as shown in Fig. 3B. At doses of 50 ng, the 13/13-mer and 12/13-mer produced on average 90.5% and 42% degradation, respectively (Fig. 3B). In all three experiments, a cleavage fragment of the expected size was seen with both antisense oligomers, and its abundance was proportional to the amount of degradation. The control oligomer produced very little degradation (below level of detection; Fig. 3B), although a faint band of a different size can be seen (Fig. 3A), indicating that a small amount of degradation did occur, but at a different site within the mRNA. In a single experiment (Fig.

3A), a control mRNA (fibronectin) was not substantially degraded ($\leq 13\%$) after injection of any of the oligomers.

It has been reported that high doses of some oligomers cause a general inhibition of protein synthesis in *Xenopus* oocytes, by an unknown mechanism (11). We therefore used ^{35}S metabolic labeling to determine whether antisense effects are accompanied by more general effects on the overall pattern of protein synthesis. Several different oligomers were tested, at doses sufficient to cause antisense degradation of their target RNAs. None of these oligomers produced reproducible changes in the pattern of protein synthesis seen on a one-dimensional gel, and in most experiments there was no effect on the overall rate of protein synthesis (data not shown). We conclude that although antisense oligomers are unlikely to be completely specific for their intended target sequences, they do not appear to cause nonspecific degradation of the majority of RNA sequences within the oocyte.

DISCUSSION

In this study, we have investigated the specificity of antisense oligodeoxynucleotides in inhibiting gene expression in *Xenopus*. We have shown that antisense oligonucleotides do not need to be perfectly complementary to their RNA target sequences in order to cleave them, and given the degree of mismatching that can be tolerated, we conclude on statistical grounds that any oligonucleotide long enough to be useful as an antisense reagent is likely to cause at least partial degradation of a large number of mRNA species.

How many RNA species are likely to be degraded? A complementary sequence of 10 consecutive bases is sufficient to produce antisense degradation, and any given 10-mer will occur about 19 times by chance in the RNA pool. Longer oligomers will contain more internal 10-mers, and we have shown that noncomplementary flanking sequences do not prevent antisense effects. Therefore, increasing the length of an oligomer beyond 10 bases is likely to increase rather than decrease the number of RNAs that will be degraded. The shortest sequence that is likely to be unique within a vertebrate mRNA pool is 13 bases, and any given 13-mer will contain 4 different internal 10-mers and will therefore recognize on average 76 different complementary sites. Furthermore, we found that a single internal mismatch within a 13-mer did not prevent antisense degradation, and a 13-mer with a single mismatch will recognize on average 12 different sites. If more mismatches, or mismatches within shorter oligomers, are permitted, the number of sites may be much larger. We have not determined the minimum length of complementary sequence needed to produce detectable antisense effects, but our data suggest that, in *Xenopus* at least, it is not possible to make an antisense oligomer that is absolutely specific for its intended target RNA.

We have assumed for simplicity that mRNA is an equal and random mixture of the four nucleotides. The actual proportions in *Xenopus* coding sequence are A:C:G:U = 0.28:0.23:0.25:0.24 (calculated from ref. 17). A target sequence enriched in C or U would therefore be slightly less frequent than our model predicts, but the effect is small; for example, a 13-mer sequence composed entirely of the least frequent nucleotide would be underrepresented only by a factor of $(0.25/0.23)^{13} = 3$, relative to the prediction of our model.

Not all oligomers are equally effective, and in all the cases we tested, perfectly matched oligomers gave more cleavage than did partially matched oligomers targeted to the same site. However, the partially matched oligomers appear to cleave at the same sites as perfect matches, since in all cases we saw the same cleavage fragments with both types. There appears to be no simple correlation between length and effectiveness; for example, an oligomer with 10 consecutive matches was more effective than other oligomers with 12/13 matches or 17

consecutive matches (Fig. 3; cf. Fig. 2), whereas another oligomer with 10 consecutive matches to Vg1 mRNA had little effect (data not shown). We do not know what determines the efficiency of cleavage, although one possibility is that accessibility of the target sequence within a ribonucleoprotein complex may be an important factor. Data from *in vitro* studies (19) suggest that the K_d for the target site will be lower for perfect than for partial matches, and since our preliminary experiments had indicated that perfectly matched oligomers were more effective than partial matches in cleaving their target RNAs, we considered the possibility that there might be a "therapeutic dose," at which we would see cleavage with perfectly matched oligomers but not with partial matches. However, our data suggest that this is not the case, since both perfect and partial matches show dose-dependent cleavage over the same dose range (see Fig. 3B). Furthermore, we note that others (20) have observed that a single antisense 14-mer causes almost complete degradation of many maternal RNA species through both perfect (14/14) and imperfect (13/14) matches. We therefore suspect that it may not be possible to achieve completely specific cleavage of a single RNA species by varying either the length or the dosage of an antisense oligomer.

The dose of antisense oligomer required for efficient RNA cleavage in *Xenopus* embryos is normally close to the toxic dose, and we suspect that at least some of this toxicity is due to fortuitous antisense effects. Consistent with this interpretation, we have found that certain oligomer sequences are especially toxic, and that this toxicity can be abolished by DNase treatment (unpublished observations; see also ref. 11); toxicity may, however, be caused by other mechanisms as well, if for instance oligonucleotides bind nonspecifically to essential components within the cell. One might predict that fortuitous antisense effects would indeed be toxic to the embryo, but in fact, many maternal RNA species can be almost completely destroyed by injection of antisense oligomers without preventing normal development (refs. 20 and 21; T.M.W. and D.A.M., unpublished data). It is therefore possible that the reason why antisense oligomers do not always kill embryos at the effective dose is simply that the majority of maternal mRNAs in the *Xenopus* embryo are not needed for normal development, perhaps because they have been sufficiently translated prior to the time of injection. Since we have never observed 100% cleavage with partially matched oligomers, another possibility is that partial degradation of many RNA species may not be deleterious to the embryo, even though complete degradation might be lethal. Indeed, genetic evidence from both vertebrates and invertebrates suggests that the levels of many gene products can be reduced by 50% without adverse effects.

How do our data relate to studies in mammalian cells and to the possible therapeutic value of antisense oligomers? It is difficult to extrapolate directly from oocytes to other cells because, unlike oocytes, most cells are constantly replacing their RNA pools, and so antisense effects will depend on rates of replacement as well as of cleavage. Furthermore, since mammalian cells grow at a temperature some 15°C higher than frog embryos, the conditions of hybridization are presumably more stringent, and so the amount of mismatched hybridization obtained may be less. Nevertheless, the possibility of fortuitous antisense effects must be considered, especially given that antisense effects have been reported in mammalian cells with as few as 11 bases (22). It is clear that to prove that a phenotype is due to the loss of a specific target mRNA, several different antisense sequences must give the same phenotype, and several control sequences must not. A number of studies have demonstrated antisense effects in cultured cells by these criteria, and in some cases, the phenotype has been rescued by injecting the depleted RNA (3, 4). Thus there is no doubt that antisense oligomers are

useful tools for studying gene function in cultured cells. However, studies in culture do not prove that antisense oligomers do not cause degradation of nontarget RNAs, even if it is shown (for instance) that cell division or total protein synthesis is not affected. Subtle gene functions are likely to go undetected in culture assays, and these may be a majority of all genes, since genetic evidence from *Drosophila* indicates that most organism-lethal mutations are not cell-lethals (23). It will therefore be important to determine whether the degradation of nontarget RNAs is a problem in whole animals. Furthermore, similar considerations may also apply to other reagents, such as ribozymes (24, 25), that cause irreversible cleavage of their target RNAs, as has been argued on theoretical grounds by others (2). If specificity proves to be a problem *in vivo*, it may be possible to overcome it by using multiple antisense oligomers to the same target RNA, each at a submaximal dose. Alternatively, it may be necessary to devise chemically modified oligomers that hybridize less strongly and therefore require longer stretches of complementarity to block gene expression.

Note. After this manuscript was accepted for publication, the paper we cite as ref. 4 was retracted (26).

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- Cohen, J. S., ed. (1989) *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression* (Macmillan, Bethesda, MD).
- Herschlag, D. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 6921-6925.
- Pan, Z.-Q. & Prives, C. (1988) *Science* **241**, 1328-1331.
- Rosner, M. H., De Santo, R. J., Arnheiter, H. & Staudt, L. M. (1991) *Cell* **64**, 1103-1110.
- Woolf, T. M., Jennings, C. G. B., Rebagliati, M. & Melton, D. A. (1990) *Nucleic Acids Res.* **18**, 1763-1769.
- Kawasaki, E. S. (1985) *Nucleic Acids Res.* **13**, 4991-5004.
- Dash, P., Lotan, M., Knapp, M., Kandel, E. R. & Goelet, P. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7896-7900.
- Cazenave, C., Chevrier, M., Thuong, N. T. & Helene, C. (1987) *Nucleic Acids Res.* **15**, 10507-10521.
- Shuttleworth, J. & Colman, A. (1988) *EMBO J.* **7**, 427-434.
- Shuttleworth, J., Matthews, G., Dale, L., Baker, C. & Colman, A. (1988) *Gene* **72**, 267-275.
- Smith, R. C., Bement, W. M., Dersch, M. A., Dworkin-Rastl, E., Dworkin, M. B. & Capco, D. G. (1990) *Development* **110**, 769-779.
- DeSimone, D. W., Norton, P. A. & Hynes, R. O. (1992) *Dev. Biol.* **149**, 357-369.
- Weeks, D. L. & Melton, D. A. (1987) *Cell* **51**, 861-867.
- Melton, D. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 144-148.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), 2nd Ed.
- Lewin, B. (1985) *Genes* (Wiley, New York).
- Aota, S.-I., Gojobori, T., Ishibashi, F., Maruyama, T. & Ikemura, T. (1988) *Nucleic Acids Res.* **16** Suppl., r315-r402.
- Melton, D. A. (1987) *Nature (London)* **328**, 80-82.
- Young, S. & Wagner, R. W. (1991) *Nucleic Acids Res.* **19**, 2463-2470.
- El-Baradi, T., Bouwmeester, T., Giltay, R. & Pieler, T. (1991) *EMBO J.* **10**, 1407-1413.
- Kloc, M., Miller, M., Carrasco, A. E., Eastman, E. & Etkin, L. (1989) *Development* **107**, 899-907.
- Chang, E. H., Miller, P. S., Cushman, C., Devadas, K., Pirollo, K. F., Ts'o, P. O. P. & Yu, Z. P. (1991) *Biochemistry* **30**, 8283-8286.
- Perrimon, N., Engstrom, L. & Mahowald, A. P. (1989) *Genetics* **121**, 333-352.
- Haseloff, J. & Gerlach, W. L. (1988) *Nature (London)* **334**, 585-591.
- Cech, T. R. (1988) *J. Am. Med. Assoc.* **260**, 3030-3034.
- Rosner, M., De Santo, R. J., Arnheiter, H. & Staudt, L. M. (1992) *Cell* **69**, 724.